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THE ADHESIVENESS OF HUMAN BLOOD PLATELETS IN VITRO

BY

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Chapter I

Introduction

A. THE ROLE OF PLATELETS IN HAEMOSTASIS AND THROMBUS FORMATION

Platelets have a key role in the initial phases of both haemostasis and thrombus formation

a) *Haemostasis*

The role of platelets in the arrest of bleeding was pointed out by Hayem (1882), and confirmed by Duke (1910) Further studies by Zucker (1947, 1949), Hugues (1953) and Roskam *et al* (1955) have shown that the formation of a platelet plug is the key event in normal haemostasis. When a small vessel is injured, the platelets start to adhere to each other and to the edge of the injury, forming a platelet plug which covers the lesion. At first this plug is permeable to the blood, but soon becomes impermeable, and the bleeding stops The change of permeability is probably due to viscous metamorphosis of the platelets (Hugues 1959 a)

b) *Thrombus formation*

Following injury to the vascular endothelium, platelets adhere to each other and to the injured point of the vessel (Hayem 1878). After a certain time the platelets which cover the injured point (the white thrombus) undergo irreversible morphological alterations (Bizzozero 1882), which were called 'viscous metamorphosis" by Eberth & Schimmelbusch (1886) Viscous metamorphosis possibly initiates or accelerates coagulation by causing release of lipids from the platelets. New platelets adhere to the fibrin network and disintegrate under the influence of thrombin (De Robertis *et al* 1953) or of thrombin together with a plasma factor (Alexander *et al* 1954) A release of more lipid follows, and a vicious circle is thus established, causing enlargement of the thrombus These observations indicate that the initial phases of normal haemostasis and thrombus for-

mation are closely related, perhaps identical. Investigations of the haemostatic mechanism are therefore important in order to obtain knowledge both of haemorrhagic conditions and of thrombosis.

B. THE ROLE OF BLOOD COAGULATION AND PLATELETS IN NORMAL HAEMOSTASIS

a) *The immediate haemostasis of freshly injured vessels*

Blood coagulation is probably of minor importance for the immediate or "primary" haemostasis This statement is based on the following facts:

1. *Fibrin is not detected in the platelet plugs which arrest the bleeding* from freshly injured small vessels (Zucker 1947). Patients with congenital afibrinogenaemia usually have a normal bleeding time (Pinninger & Prunty 1946, Frick & McQuarrie 1954, Alexander *et al.* 1954). Technical errors are thought to be responsible for the prolonged bleeding time reported in a few cases (Frick & McQuarrie 1954, Soulier *et al.* 1955).

2. *Patients with congenital defects in coagulation factors* have a normal bleeding time (Borchgrevink & Waaler 1958).

3. Dicoumarol treatment of rats does not prevent the formation of the platelet plugs following injury of the small vessels (Zucker 1947). Dicoumarol treatment of rabbits caused only a slight prolongation of the bleeding time in 50 per cent of the animals (Cauwenberge & Jaques 1958). Excessive treatment with phenylindanedione in man (PP value less than 5 per cent) gives only a slight prolongation of the bleeding time (Borchgrevink & Waaler 1958).

4. Heparin in *ordinary* therapeutic doses, did not prevent the production of white thrombi in rats *in vivo*, nor did it prevent the formation of thrombi in arterio-venous glass shunts (Zucker 1947). In rabbits, platelet plugs formed in the mesenteric vessels after injury in the presence of about 0.003 per cent heparin, a concentration somewhat higher than the therapeutic concentration commonly used in man (Roskam *et al.* 1955). When bleeding from the small blood vessels in the hamster cheek pouch was provoked with a microelectrode, treatment with heparin, dicoumarol, Tromexan and phenylindanedione enhanced the formation of a platelet plug at the site of haemorrhages (Fulton *et al* 1953).

In man only a slight prolongation of the bleeding time within the normal range could be demonstrated after heparin treatment (Hjort & Stormorken 1957, Borchgrevink & Waaler 1958). In mice, following *massive* doses of heparin, the bleeding time was prolonged only in some of the animals (Copley & Lalich 1942).

5. Injection of hirudin in geese and dogs in doses sufficient to inhibit blood coagulation did not prevent thrombus formation *in vivo* (Loeb 1906).

6. In certain arthropods (*Limulus*) no fibrin is detectable in the blood, and "haemostasis" is due solely to a plug formed by *amoebocytes* (Loeb 1906). By comparative phylogenic studies it was found that "haemostasis" in lower species is based on cellular aggregation only. In higher species an additional mechanism, coagulation, has been developed. The cellular mechanism, however, always precedes the coagulation (Silberberg 1938).

There are, however, some reports which suggest an influence of blood coagulation on the "primary" haemostasis.

1. Injection of *large* doses of heparin in rats prevented the formation of platelet plugs *in vivo* (Zucker 1947). Large doses of heparin also prevented the formation of thrombi in arterio-venous glass shunts in dogs, cats and monkeys, but not in rabbits (Best *et al.* 1938). Doses less than 300 U/kg, however, did not prevent the thrombus formation in dogs (Solandt & Best 1940).

2. *In vitro*, platelet aggregation and viscous metamorphosis are provoked by certain coagulation products (Bergsagel 1956, Bounameaux 1957, Sharp 1958, Biggs *et al* 1958, Waaler 1959 a).

b) *The maintenance of haemostasis*

Blood coagulation may therefore not be necessary for the immediate haemostasis but it is essential for the maintenance of haemostasis. Thus, patients with coagulopathies usually have a normal bleeding time, but a haemorrhagic diathesis which is characterized by "late" or "after" bleedings. This observation indicates a difference between the process which arrests bleeding in freshly injured vessels and the process which secures a permanent haemostasis. Such differences in the mechanisms of the "primary" and the permanent haemostasis have recently been demonstrated by Borchgrevink & Waaler (1958). They measured the bleeding time in patients with different coagulopathies. After 24 hours, bleeding was provoked by ently removing the crust from the wound of the previous determination, nd the bleeding time was measured again at this site. This was called "the condary bleeding time" in contrast to the bleeding time of the fresh ound, which was called "the primary bleeding time". Patients with rombocytopenia had prolonged both "primary" *and* "secondary" eding times. Patients with defects of the coagulation factors belonging the "intrinsic system" had a normal "primary", but a prolonged ondary" bleeding time.

These investigations strongly suggest that platelets are responsible for the primary haemostasis, whereas both platelets and the coagulation factors belonging to the intrinsic clotting system are responsible for permanent haemostasis in man.

C. THE PLATELET ADHESIVENESS

The adhesion of platelets to an injured vascular wall or to a foreign surface has been called *adhesion*, *aggregation*, or *agglutination* by different authors. According to Braunsteiner (1955), the property of the platelets to adhere to certain foreign surfaces is called platelet *adhesiveness*. This process is associated with morphological alterations of the platelets, including formation of pseudopods, spreading out of the hyalomere and coalescence of the granulomere to a central pseudonucleus Braunsteiner & Pakesch (1956) strictly distinguished between *adhesiveness* and the ability of the platelets to stick to each other in the absence of immunological mechanisms *(aggregation)*. When platelets formed clumps as the result of a reaction between antigen and antibody, they used the term *agglutination*.

Lundevall (1958) used the expression "*unspecific clumping*" instead of aggregation.

In this work, the terminology of Braunsteiner (1955) is used, but a strict differentiation between adhesiveness and aggregation of platelets is not accepted for the following reasons:

1. Aggregation of platelets *in vitro* may be provoked by different agents It is the initial step of viscous metamorphosis and has been supposed to be induced by some coagulation factors and calcium (Wright & Minot 1917), by Product I (Bergsagel 1956), by PTA (Sharp 1958), by PTA and Hageman factor (Biggs *et al* 1958), or by "Activation product" (Waaler 1959 a) Platelets may also be aggregated by different bacterial toxins, snake and bee venoms, histamine and heparin, further by $HgCl_2$, $HgCN_2$, saponin (Oellgaard 1943), or by a substance in the red cells (Oellgaard 1951) Washed platelets in saline are aggregated at pH above 7 in the presence of calcium (Lundevall 1958).

2. The first step in normal haemostasis includes both adhesion of platelets to the vascular wall and platelet aggregation Both processes occur simultaneously and immediately after the vessel injury. The basic mechanisms are therefore probably identical, but not related to any activation of the coagulation process, because such activation needs at least several seconds

D. THE MECHANISM OF THE ADHESION OF PLATELETS TO THE VASCULAR WALL OR TO FOREIGN SURFACES

This mechanism is poorly understood and several theories have been proposed:

1. The adhesion is due to alteration of the electrical charge of the injured intima (Sawyer *et al.* 1953 a, b, c).
2. The platelets are mechanically injured by contact with the "foreign surfaces", and therefore become adhesive (Loeb 1906).
3. The adhesion is due to alteration ("opsonisation") of the injured intimal surface. The role of the platelets is passive and independent of viability (Roskam 1922-3).
4. Platelet adhesion depends on the deposition of a fibrin film on their surface (Wright 1945).
5. Thrombin formation, together with certain cofactors, makes the platelets adhesive (Bounameaux 1957) This hypothesis cannot, however, explain why platelet adhesion occurs immediately after injury.

There is evidently no agreement as to the mechanism of platelet adhesion, probably because the methods for investigation of platelet adhesiveness are still unsatisfactory. In spite of the major importance of platelet adhesiveness in haemostasis and thrombus formation, surprisingly few investigations of this problem have been reported This fact calls for further work in this field since increased knowledge of platelet adhesiveness may shed light on the mechanism both of thrombus formation and of haemostasis.

E PLAN OF THIS STUDY

The principle aim of the present work has been to study the mechanism of the "immediate" adhesion of human platelets to foreign surfaces and the factors which influence this process. The plan also included a study of the platelet adhesiveness in normal subjects and in patients with bleeding disorders This was done in order to determine the normal range and to investigate a possible correlation between the adhesiveness of platelets and their haemostatic effect Due to technical errors inherent in the methods previously reported, a more reliable method for estimation of platelet adhesiveness had to be developed

Materials, methods and definitions

1. MATERIALS

Cannulas. Siliconized steel cannulas, gauge 19, were used for withdrawal of blood.

Cephalin. A crude cephalin suspension was prepared by the technique described by Hjort *et al.* (1955).

Citrated whole blood. Normal whole blood was collected in siliconized glass tubes containing 1/10 volume of 3.13 gm per cent trisodium citrate dihydrate If the haematocrit was outside the normal range (40-50), the volume of citrate was adjusted according to the haematocrit value to give a constant citrate concentration in the plasma (p. 18).

Citrated platelet-rich plasma. Citrated whole blood was centrifuged for 15 minutes at 190 G at 4° C. The plasma was collected with a siliconized pipette

Citrated platelet-poor plasma. Citrated platelet-rich plasma was centrifuged for 20 minutes at 81,000 G at 4° C in a "Spinco" centrifuge, model L. The supernatant contained less than 700 platelets per c.mm.

Counting chambers. The counting chamber of Bürker was used for platelet counting.

Chromo-sulphuric acid solution was prepared by dissolving 15 gm of anhydric chromic acid, c.p. (Merck, Darmstadt, Germany) in 800-1,000 ml of distilled water. To this solution was added 5 l of concentrated sulphuric acid.

EDTA. A 4 5 per cent (w/v) solution of ethylene-diamine-tetraacetate dihydrate ("Komplexon III" from Siegfried, Sofingen, Switzerland) in distilled water was used as a stock solution. The solution was adjusted to pH 7.3 with N/2 NaOH.

Glass bead column (filter). Plastic tubing about 20 cm long with an internal diameter of 5 mm was used for preparation of a column. *A filter unit* was inserted in the lower end. The filter unit was prepared from two

plastic tubings, one with an internal diameter of 2 mm and external diameter of 3 mm, the second with an internal diameter of 3 mm and external diameter of 5 mm. A sieve cloth of silk (GG 48, Dufour & Comp., Thal, Switzerland) covered the upper end of the filter unit (see Fig. 2).

Five gm of glass beads (Reflex Perlen 31/7, Dragon-Werk, Bayreuth, Germany) were poured into the tubing. The tubing was then cut 4 mm above the upper layer of the beads. Each column contained about 15,000 beads. Finally a filter unit was inserted in the upper end of the tubing. To avoid any leakage, the filter units were cemented to the main plastic tubing with "Casco plast folie lim" from A/S Norcasco, Oslo, Norway.

Glass tubes. Siliconized glass tubes, 15 × 100 mm, calibrated for 10 ml were used for collection of blood samples.

Heparin. Heparin 5 per cent ("A-L", Oslo, Norway) containing 5,000 I.U. per ml was used.

Ion exchange resins. Anion exchange was performed with Dowex 2, 200-400 mesh (Dow Chemical Co., Midland, Michigan, U.S.A.). The resin was treated successively with 10 volumes of N/1 NaOH, 2 volumes of NH_4 formate, 10 volumes of N/1 formic acid, and finally washed with distilled water until the reaction of the washwater became neutral. The cation exchange was performed with Dowex 50, 200-400 mesh. The resin was treated successively with 10 volumes of N/10 HCl, 2 volumes of H_2O, 10 volumes of a 10 per cent solution of NH_3, 2 volumes of H_2O, 10 volumes of N/10 HCl, and finally washed with distilled water until neutral reaction of the washwater.

"Parafilm" from Marathon Corp., Menasha, Wisconsin, U.S.A., was used as a non-wettable cover for tubes during mixing procedures.

Phase contrast microscope (Zeiss Photomicroscope) supplied with a light green filter (type FAL, Jenaer Glaswerk, Schoott & Gen., Mainz, Germany) was used for platelet counting and for photography of platelets.

Saline. A 0.9 per cent solution of sodium chloride in distilled water was used. In some experiments (which are mentioned in the text) other concentrations were used.

Silicone. A 0.5 per cent solution of "Monocote" (Armour Laboratories, Eastbourne, England) was used for the steel cannulas. A 2 per cent solution of DC 1107 (Dow Corning Co., Midland, Michigan, U.S.A.) was used for the glass ware. A 10 per cent solution of "Siloxan 300" in ethyl ether (Uddeholm AB, Uddeholm, Sweden) was used for the syringes.

Syringes. A 5 ml siliconized syringe was attached to the mechanical device. The device delivered exactly 1 ml of blood from the syringe in 23.5 seconds. The time was controlled with a stop watch.

Thromboplastin. A saline extract of human brain was prepared according to Hjort (1957, p. 17).

Triton. A solution of 1 per cent Triton (Winthrop Laboratories, New York, U.S.A.) in saline was used.

Trypsin. "Trypure Novo" (Novo Terapeutiske Laboratorium, Copenhagen, Denmark), containing 22-25 Anson units of trypsin per gm dry-frozen substance, was dissolved in phosphate buffer (pH 7.7) to give a 1 per thousand solution.

2. METHODS

Antihaemophilic A factor assay was performed with the method of Waaler (1959 b).

Antihaemophilic B factor assay was performed with a one-stage cephalin method described by Stapp (1958).

Bleeding time. A modified Ivy technique was used (Borchgrevink & Waaler 1958). The normal range is 2-12 minutes.

Cephalin time was estimated as described by Waaler (1957).

Centrifugation. An "International" refrigerated centrifuge (model PR-2) was used. The maximal G[1] when using 10 ml tubes was about 200 at 1,000 r.p.m. and about 1,800 at 3,000 r.p.m. For high speed centrifugation the "Spinco" ultra centrifuge, model L, was used. The maximal G was about 9,000 at 10,000 r.p.m., about 36,000 at 20,000 r.p.m., and about 81,000 at 30,000 r.p.m. The material was centrifuged in lusteroid tubes at about 4° C.

Dialysis was performed in Schaldarm casings (Kalle & Co., Wiesbaden, Germany).

Cleaning procedures for syringes and glass tubes are described on p. 16 and for the counting chambers on p. 21.

Ion exchange experiments with Factor R were performed in Erlenmeyer flasks as described in the text (p 73). Columns were not used, because the solution of Factor R passed through the resin column too slowly. Before the anion exchange the solution of Factor R was adjusted to pH 9 with NH_4OH In the cation exchange experiments, the solution was adjusted to pH 2 with HCl.

Platelet adhesiveness assay was performed with the author's method. The equipment is described in the text (p. 14) The practical performance of the assay is described on pp 17-19.

[1] Relative Centrifugal Force = 1118 x R_{max} x (r.p m)^2 x 10^{-8}.

Platelet counts. A slight modification of Nygaard's method (1933) was used; see p. 21.

P & P estimation was performed as described by Owren & Aas (1951).

Prothrombin was estimated by the method of Hjort *et al.* (1955). This method is sensitive to both prothrombin and Stuart factor.

Proconvertin was estimated by the method described by Aas (1952).

Proaccelerin was estimated by the method described by Aas (1952).

Silicone coating of syringes, glassware and cannulas is described in the text (p. 16).

3. DEFINITIONS

Factor R is a substance released from the stroma of the red cells. Added to platelet-rich plasma, it increases the adhesiveness of viable platelets.

Platelet adhesiveness is the property of platelets to adhere to foreign surfaces. It is measured quantitatively as the actual number, or the percentage, of platelets which are lost when blood, or platelet suspensions, are allowed to pass a standardized glass bead column.

Viscous metamorphosis is defined according to Wright & Minot (1917): "The platelets begin their metamorphosis by aggregating and agglutinating in masses and strands which increase in size up to a certain point within a few minutes. Then within a few more minutes the platelets in these masses and strands lose their high refrangibility, seem to fuse one with another and their separate identity becomes lost or obscure. Some contraction of the volume of the masses and strands may occur during the process"

The assay of platelet adhesiveness

1. PREVIOUS METHODS FOR ESTIMATION OF PLATELET ADHESIVENESS

Baar & Szekely (1929) estimated the decrease of platelet number in plasma after storage of the plasma in glass containers Baar (1941) claimed this to be the first test devised for platelet function In this test, however, the decrease of platelets was partly due to adhesion of the platelets to the glass wall and partly caused by disintegration of the platelets

Morawitz & Jurgens (1930) constructed an apparatus ("Kapillarthrombometer") consisting of 2 pumps which drew blood without anticoagulant through a calibrated glass capillary tube The direction of the blood stream was alternated at intervals After a certain time the blood stream ceased, due to plugging of the capillary tube by platelets. This time ("Thrombosezeit") was taken as an indirect measure of the adhesiveness of the platelets In all probability, the coagulation of the blood also influenced the results.

Methods have been described for the study of experimental extracorporeal thrombosis (Rowntree & Shinoya 1927, Johnson 1927, Best *et al.* 1938). The principle of these methods is to shunt the blood stream from arteries to veins through tubes or chambers, in which thrombi are formed after a certain time. When flat chambers were used, the formation of platelet thrombi was easily detected by microscopic investigation of the chambers The time required for formation of white thrombi, or for plugging the tubes, gave a rough estimation of the platelet adhesiveness These methods are semiquantitative

The first real attempt to measure the adhesiveness of platelets quantitatively was made by Wright (1941). The principle of the method was as follows: Blood mixed with anticoagulant was transferred to a special glass tube, whose sides had been ground away to form two windows about 1.5 cm in diameter. The windows were covered with uncoated glass, while the

internal surface of the bulb was coated with melted vaseline. The tube, containing 2 ml of blood, was rotated at 3½ r.p.m. Every twenty minutes platelet counts were made in samples withdrawn from the tube, and the decrease in platelet number was recorded A similar tube coated all over with vaseline served as control. The uncoated glass area, in this method, seems to be too small, and therefore the time of procedure had to be long (up to 80 minutes). This might cause errors by platelet disintegration (Baar 1941).

In order to shorten the time of contact between platelets and glass surface, Moolten & Vroman (1949a) described a method in which a glass-wool filter served as the foreign surface. Commercial glass-wool was drawn into 3 strands of equal size and worked into a loose braid 0.5-1 cm thick and 6-7 cm long. Before use, the glass-wool filter was moistened with 1 5 ml of physiological saline. One ml of citrated blood was deposited in the braid Exactly 30 seconds later the blood was washed through with 8 ml of normal saline, containing 1/10 its volume of isotonic citrate solution. The red cells and platelets were counted in the blood sample before and after the filtration The adhesiveness of the platelets was computed from the ratio red cells/platelets in both samples

There are two sources of error in this method Firstly, the glass-wool filter is prepared in such a way that the area of glass surface cannot be standardized, secondly, the use of saline and saline-citrate solution changes the adhesiveness of the platelets. Even platelets from patients with thrombasthenia become sticky when suspended in saline (Braunsteiner 1955, confirmed in the present work, pp. 86-87).

Rovatti (1951) used the capillary part of a red cell counting pipette as foreign surface A) From an incision in the finger tip, blood was allowed to drip into a container in which 12 per cent magnesium sulphate was deposited in order to prevent coagulation and abolish the platelet adhesiveness The red cells and the platelets in this solution were counted, and the ratio red cells/platelets determined B) With a second pipette, an ordinary count of the red cells was performed on the blood from the incision. C) Blood from the incision was drawn to the 0 3 mark in a third pipette The blood column was then drawn to the 1 mark and subsequently blown down to the 0 3 mark. Finally the blood was diluted with a solution of magnesium sulphate to the 101 mark, and the platelets counted From the values obtained by the procedure A and B, the "true" value of platelets in the circulating blood was computed The difference of the calculated number and the number of platelets found in the third pipette gave the number of adhesive platelets In this method the glass area is too small, and

the time of contact between platelets and glass surface is not standardized. The calculation of the number of adhesive platelets involves four countings, which considerably decreases the accuracy of the method.

In the method of Revol (1954) platelets were not counted. Ten ml of venous blood was collected in a tube containing 1 drop of heparin and 0.5 ml of a 4 per cent citrate solution. The blood was centrifuged for 3-4 minutes at 3,000 r p.m. One drop of the platelet-rich plasma was deposited between a slide and a cover-glass and examined with oil immersion lens in a phase contrast microscope. After 10 to 60 minutes, normal platelets *spread out and adhered to the glass surface.* "*Pathological platelets formed* dendrites as normal platelets, but did not spread out or adhere to the glass " This method is not quantitative.

Marx & Derlath (1957) described a method in which a capillary tube with a length of 500 mm and a bore of 1.174 mm was used as foreign surface. One tenth ml of citrated blood was deposited in the tube, and the tube was tilted to an angle of 60°. When the blood column in the capillary tube had run exactly 400 mm, the tube was tilted to the opposite side. This was repeated for 5 minutes, and the number of tiltings gave a relative value of the viscosity of the blood. The difference in the platelet number before and after the procedure was the number of adhesive platelets. The test was performed at 37° C. The contact area in this method varies considerably with the viscosity of the blood if the contact time is kept constant. If the contact area is kept constant by letting the blood run a constant distance in the tube, the contact time varies with the viscosity of the blood.

2. THE PRESENT METHOD FOR DETERMINATION OF PLATELET ADHESIVENESS

A. *Theoretical considerations*

Remarks made in the preceding chapter illustrate the need for better methods for determination of platelet adhesiveness. An *in vivo* technique would be the most satisfactory way of studying the adhesiveness of platelets from a physiological point of view, but such a method is probably impracticable with humans Any *in vitro* method inevitably introduces at least two unphysiological factors, namely, an anticoagulant and a foreign surface. The choice of anticoagulant will be discussed later (pp 33-36). As a foreign surface glass is convenient because it is easily available and easy to clean by standardized procedures.

A reliable *in vitro* method requires the following conditions. The amount of anticoagulant in the plasma must be constant. It is therefore necessary to adjust the amount of anticoagulant added to the whole blood in relation to the haematocrit. The area of foreign surface must be constant, and its physico-chemical properties must be standardized. The time of contact between platelets and the foreign surface must be constant and of a suitable duration. The time between withdrawal of blood and the start of contact with the glass surface must be constant.

B. *Principle of the method*

Estimation of platelet adhesiveness is done in citrated blood with a constant concentration of citrate in the plasma. By an electrically driven mechanical device, the blood is pushed from a graduated syringe through a standardized glass bead column at a constant rate. The reduction in platelet count after passage through the glass bead column is taken as a measure of platelet adhesiveness. It is consequently assumed that the reduction in platelet number by the passage is caused by adherence to the glass beads.

Theoretically, platelet loss might also be caused by platelet disintegration, by adhesion of platelets to fibrin strands, or by formation of platelet clumps which are mechanically arrested by the column. These causes for platelet disappearance are, however, unlikely for the following reasons. The disintegration of platelets in isotonic solutions requires a considerable time (Tullis 1953). When citrated platelet-rich plasma is clotted with thrombin, disintegration of platelets does not occur for several minutes (De Robertis *et al.* 1953). As the contact time in the present method is only 30 seconds, disintegration by prolonged contact with thrombin is very unlikely.

By microscopic examination of the beads after the passage of blood neither fibrin nor platelet aggregates were observed.

Presumably, optical difficulties prevented the detection of platelets actually adhering to the glass beads. The adherence of platelets to plane glass surfaces was, however, readily observed by the following method. A drop of citrated blood was placed on a cover slip, carefully agitated for a few minutes, and washed off with plasma. A thin greyish film remained on the slip. This film consisted of large numbers of adhered platelets, as shown in Microphotograph no. 1. The platelets adhered to this surface, and there is no reason to suppose that platelets do not adhere to the curved surfaces of beads (surface area of one bead $= 785{,}000\ \mu^2$) in the same way, which is the principle of the present method.

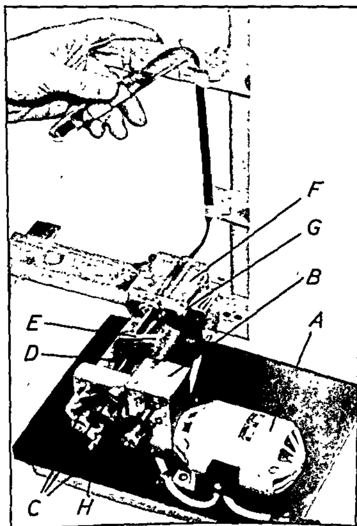
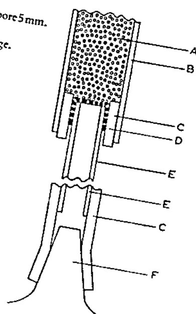


Fig. 1. *The apparatus used in the method for estimation of platelet adhesiveness* (For description, see text)

C. *Equipment*

1. *The mechanical d*[illegible] vering [illegible] a constant rate consists of the following [illegible] 1). A [illegible] v motor with a speed of 2,900 r.p m. (A). [illegible] spe[illegible] rotation is [illegible] p m [illegible] the movement i[illegible] to [illegible]),

Fig. 2 *Diagram of the glass bead column.*
A Glass beads, diameter 0 5 mm. B. Plastic tubing, bore 5 mm.
C. Plastic tubing, bore 3 mm D Sieve cloth of silk.
E. Plastic tubing, bore 2 mm F Conus of the syringe.



(E) for the plunger of the syringe The syringe is kept in position in the groove of the brass block by a transparent plastic cover (F). An adjustable microswitch (G) automatically turns off the motor just before the syringe is empty The interchangeable gears were used only for the methodological studies, and are unnecessary for routine investigations A hand-driven crank (H) is used for reversing the adapter for the syringe plunger.

2 *The glass bead filter*, as described earlier (p 6) consists of 5 gm of glass beads in plastic tubing Both ends of this column are provided with "filter units" to prevent the loss of beads from the tubing. Each of the glass beads has a diameter of 0 5 mm and each filter contains about 15,000 beads Thus the surface area may be calculated to be about 12,000 mm² Since a large number is used, the error due to minor differences in diameter of the beads is minimal, and the surface area is relatively constant.

The blood is forced through the glass bead column by the mechanical device, which delivers blood at a constant rate The time required to deliver one ml of blood is called *the delivery time* The volume within the column which is not occupied by beads amounts to 1 28 ml The time required for a platelet to pass through the column is therefore *the delivery time* × 1.28. This time is called *the contact time* With a delivery time of 23.5 seconds, the contact time is 23 5 × 1 28 = 30 seconds In the following the dif-

ference between *delivery time* and *the contact time* must be kept in mind to avoid confusion.

3. *Other requisites* used in the method are described in chapter II (Materials).

D. Preparation of the equipment

The mechanical device was constructed and built by The Central Institute for Industrial Research, Blindern, Oslo, Norway.

The glass bead filters are prepared from three different sized plastic tubes (see Fig 2). The external diameter of the smaller tube equals the internal diameter of the larger tube. The medium and the small sized tubing is used for preparation of the "filter units" which close both ends of the largest tubing. It is important to avoid leakage of blood from the glass bead filter. Plastic glue is therefore used to seal the connections.

The steel cannulas are boiled for 3 minutes in a solution of "Monocote" and rinsed in running tap water for 20 minutes, followed by rinsing twice with distilled water. After dry sterilization at 180° C for 180 minutes, the cannulas are ready for use.

The syringes are cleaned in a solution of sodium carbonate, rinsed several times in tap water and distilled water, and dried. The syringes are then filled with a 10 per cent solution of siloxan in ethyl ether. After one minute they are emptied and allowed to drain for 15 minutes, followed by heating to 180° C for one hour. This procedure gives a greasy film, with excellent water repellent property, which does not inhibit the movement of the piston.

The glass tubes are cleaned with a solution of sodium carbonate, rinsed in tap water, and kept in a solution of chromo-sulphuric acid for 24 hours. After rinsing in tap and distilled water and drying, the tubes are filled with a 2 per cent solution of DC 1107 for two minutes and drained. After heating to 180° C for one hour, they are ready for use. All siliconized equipment is cleaned and resiliconized each time it is used.

The glass beads are soaked in a solution of sodium carbonate for 2 days. After rinsing in running tap water for half an hour, they are soaked in chromo-sulphuric acid solution for two days, and rinsed in running tap water for 2 hours with occasional stirring, followed by rinsing in large quantities of distilled water. The beads are dried at 120° C for 24 hours It must be stressed that large quantities of water for rinsing are necessary, as otherwise the beads will retain too much chromate on their surface.

E. Technique for estimation of platelet adhesiveness by the present method

The following is a description of a double test. The patient's haematocrit was estimated. At normal haematocrit (40-50 per cent) one ml of a 3.13 per cent citrate solution was pipetted into a siliconized glass tube (100 × 15 mm), supplied with a mark at 10 ml. A siliconized needle was used for the venepuncture After the first 2-3 ml of blood were discarded, the tube was filled to the 10 ml mark. The tube was covered with Parafilm and inverted to mix the contents Twelve minutes later, the blood-citrate mixture was transferred to two siliconized 5 ml syringes. A plastic tubing, 5 cm long with a bore of 2 mm, was attached to each syringe.

One syringe was placed in the brass block of the motor-driven device. The interchangeable gears of the motor had been adjusted to give an outflow of 1 ml blood from the syringe in exactly 23.5 seconds. The motor was switched on, and a stop watch was started when the blood reached the end of the outflow tubing of the syringe The blood was allowed to drain for exactly 23 5 seconds into a glass tube (150 × 15 mm) which contained 19 ml of a 3 13 per cent citrate solution. The end of the tubing was held about 1 cm above the level of the citrate solution. The glass tube now contained a mixture of 19 ml of citrate solution and 1 ml of citrated blood, which had *not* passed the glass bead column. The tube was inverted 10 times and set aside The syringe was then connected with the glass bead column, which was kept vertically in a stand. Exactly 15 minutes after withdrawal of the blood the motor was again started. When the blood had passed the column and reached the end of the outflow tubing, the stop watch was again started, and the blood was collected in a second tube containing 19 ml citrate solution. The motor was stopped 23.5 seconds later The second collection tube now contained 19 ml of citrate solution and one ml of citrated blood, which *had* passed the column. The tube was inverted 10 times and set aside for sedimentation of the red cells.

The procedure was repeated with the second syringe

After sedimentation of the red cells for 2-3 hours, the platelets in the four supernatants were counted The mean of the difference in the two parallel tests gave the number of adhesive platelets per c.mm. This value was also recorded as per cent of the total number of platelets in the blood

The test was always performed in citrated blood, and the number of platelets recorded always refers to citrated blood. It is 9/10 of the number of platelets in "native" blood.

F. *Adjustment of the amount of citrate added at abnormal haematocrit values*

Wright (1941) showed that the adhesiveness of platelets decreased with increasing concentration of oxalate, heparin and chlorazol dyes. As shown in the present work (p. 33) the addition of increasing amounts of citrate to the blood has the same effect. The concentration of citrate in plasma must therefore be kept constant. The citrate solution must be isotonic, otherwise the adhesiveness of platelets is changed (pp. 43-46).

The necessary amount of citrate solution which must be added to give a constant concentration of citrate in plasma at different haematocrit values was calculated as follows:

The final volume of the citrate-blood mixture is always 10 ml The amount of citrate added is X ml The amount of blood is 10 - X.

$$\text{The amount of plasma} = \text{ml whole blood} \cdot \frac{100 - H}{100}$$

$$= (10 - X) \cdot \frac{100 - H}{100}$$

At normal haematocrit (45 vol per cent), 9 ml of blood is mixed with 1 ml of isotonic citrate solution. The ratio citrate/plasma is then 1/4.95. This ratio has to be kept constant at any haematocrit value,

$$\frac{X}{\text{amount of plasma}} = \frac{1}{4.95}$$

$$\frac{X}{(10 - X) \cdot \frac{(100 - H)}{100}} = \frac{1}{4.95}$$

$$495\,X = (10 - X)\,(100 - H) = 1{,}000 - 10H - 100X + HX$$

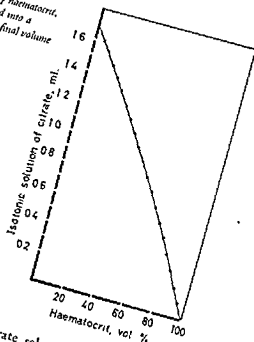
$$X(595 - H) = 10(100 - H)$$

$$X = 10 \cdot \frac{(100 - H)}{595 - H}$$

Using this formula, the ratio citrate/plasma is 1/4.95 at any haematocrit value.

The values of X at different haematocrit values were calculated and plotted on a graph (Fig 3). In later experiments, the amount of citrate to be added was read from this graph.

Fig. 3. *Graph used to obtain a constant concentration of citrate in plasma at any haematocrit. The correct amount of citrate is pipetted into a siliconized tube and blood is drawn to a final volume of 10 ml*



For practical use, the amount of citrate solution was corrected for haematocrit values above 50 and below 40 vol per cent, whereas no corrections were made for values between 40 and 50 vol per cent. Any correction of the amount of citrate solution introduces an error in the estimation of the platelet count. At a haematocrit value of, say, 20 vol per cent, 1.39 ml citrate solution are added to 8.61 ml of blood. The platelet count is then somewhat lower than if the ordinary dilution of 1:10 had been used, and the number obtained should be multiplied by the factor 9/8 61 = 1 04 to give the correct value As the technical error is great in platelet counting, this error of 4 per cent was, however, ignored

G. *The counting of platelets*

a) Remarks on methods

The counting of platelets is a key problem in the present work. All currently used methods have a relatively great error If 1,000 platelets are counted in each of two chambers, the expected coefficient of variation is calculated to be 3 86 per cent Thus within 95 per cent confidence limits the true value is equal to the observed value ± 7 72 per cent (see p 23), which represents minimum error.

More than 150 methods for the counting of platelets have been described (Maupin 1954, p. 10). The different principles and a few examples of the commonly used methods will be discussed. The methods may be divided into three groups.

1) *Indirect methods:* In these the ratio of platelets to red cells is estimated by counting the number of platelets and red cells in either a blood smear or in a counting chamber after suitable dilution of the blood. From this ratio and an ordinary red cell count, the number of platelets is calculated. In healthy males with a ratio of 1:20 the minimum error is 14 per cent when platelets among 1,000 red cells are counted in a chamber. In addition, a red cell count, which has an error of about 8 per cent, is required to obtain the platelet number per c mm. The combined errors are $\sqrt{14^2 + 8^2} = 16$ per cent (Brecher *et al.* 1953). Thus, the counting error in indirect methods is always greater than in direct methods.

When smears are used for estimation of the ratio platelets/red cells, a higher ratio is usually found. According to Brecher *et al.* (1953), the reason is probably a tendency to pick out areas where platelets are readily seen, *i. e.* areas with a high number of platelets. The highest counts recorded are not necessarily correct (Wintrobe 1951, p. 241).

2) *Direct methods.* In these the blood is mixed with a solution containing an anticoagulant, and which either a) preserves both the red cells and platelets, or b) haemolyses the red cells and preserves the platelets The platelets are counted in a counting chamber after suitable dilution of the blood.

An example of the first principle is the method of Rees & Ecker (1923); examples of the second are the methods of Feissly & Lüdin (1949), and of Brecher & Cronkite (1950).

3) *Plasma platelet count* The counting of platelets in plasma following sedimentation of the red cells was first done by Thomsen (1920). He used blood mixed with 1/10 the total volume of a 10 per cent solution of citrate. After the red cells were allowed to sediment, the platelet-rich plasma was diluted with normal saline and the platelets were counted in a counting chamber. To determine the platelet numbers in whole blood, the haematocrit was used as a correction factor

Nygaard (1933) described a modification of Thomsen's method in which blood was diluted 1·20 with a 1 6 per cent sodium oxalate solution prior to sedimentation After sedimentation of the red cells, the platelets in the supernatant were counted This 20-fold dilution of the blood greatly reduced the error caused by variation of the [illegible] In Nygaard's method, the observed platelet [illegible] in b[illegible] with haema-

tocrit values of 20 and 80 vol per cent are respectively only 1 and 4 per cent higher than the correct value. Correction for abnormal haematoc[rit], therefore, can be neglected in most instances.

b) Choice of method for platelet counting

The author has tested three methods for platelet counting: The methods of Feissly and Lüdin, Brecher & Cronkite, and Nygaard. Nygaard's method, slightly modified, was chosen for the following reasons: 1) The use of micropipettes was avoided, 2) The number of platelets in the supernatant remained constant for about 6 hours, which was important when investigating the large series in this work. 3) The use of citrate solution as a diluent preserves the morphology of the platelets for hours, and gives a clear picture in the phase contrast microscope of an almost pure suspension of platelets (microphotograph 2).

The method of Feissly & Ludin, and that of Brecher & Cronkite were rejected because the large number of red cell ghosts made platelet counting both difficult and tiresome when counting large series (microphotographs 3 and 4). It was further found that in the method of Brecher & Cronkite many blood cells were broken into small particles if the pipettes were shaken too vigorously (a pipette rotor was not available). This was especially true when the blood had been mechanically injured by passing through the glass bead column. Thus the counting of platelets was often difficult or even impossible, particularly if a dilution of 1:20 were used (microphotograph 5)

c) Platelet counting by Nygaard's method

One ml of blood was mixed with 19 ml of 3.13 per cent citrate solution in a 150 × 15 mm siliconized tube. The tube was inverted gently 10 times, and allowed to stand on the desk for 2-3 hours for sedimentation of the red cells to occur. A Burker counting chamber was filled with a small sample of the supernatant, and great care was taken to avoid overflow. This was then placed in a moist chamber, and the platelets were allowed to sediment during 30 minutes. At the end of that time about 1,000 platelets were counted with a phase contrast microscope, using 400 times magnification.

Errors due to the use of improper technique can be avoided. The chambers must be completely clean. This was achieved by washing the chambers immediately after use with a solution of sodium carbonate and a smooth brush. After rinsing first with tap and then distilled water, the chamber

was dried with a soft cloth. Just before use, the chamber again was wiped with a smooth linen cloth to remove any dust. Prior to filling, the empty chamber was inspected under the microscope for the presence of dust particles. Another error arises if the cover glass is touched, for example by the microscope's objective, after sedimentation of the platelets In this instance the platelets run together in the centre of the chamber and become unevenly distributed.

All methods also involve the error caused by counting fragments of red cells (Arnold bodies), bacteria, yeast and dust as platelets. This source of error is usually avoided in Nygaard's method except when studying blood from patients with severe anaemia, where numerous microcytes and/or Arnold bodies may occur together with the platelets in the supernatant. Normal platelets appear as more or less dark sphaerules on a bright background, depending on the type of phase contrast microscope used They are 1-2 μ in diameter and have one or two delicate spicules. Occasionally platelets with a diameter of about 3 μ or even more may occur. These platelets are pink, like the red cells or fragments of red cells. Microcytes and Arnold bodies, however, lack spicules. Difficulties in recognition of platelets therefore do not play any important role in the error of counting.

Another source of error in Nygaard's method might be sedimentation in the glass tube of aggregated or large platelets, resulting in too low values. The mean count in *citrated blood* (1:10) from 72 normals was 218,000 per c.mm (see Table 1), which equals 241,000 per c.mm in whole blood. These figures agree with the results of Brecher & Cronkite (1950), who counted platelets in 50 normal young men, using capillary blood. They found a mean of 257,000 platelets per c.mm, and a range of 87,000-498,000 platelets per c mm Sedimentation of agglutinated platelets therefore does not seem to be a source of error in Nygaard's method.

H. Precision of the method

According to Berkson et al. (1940) the coefficient of variation of a platelet count in a haemocytometer is given by the following formula:

$$V_t = \sqrt{\frac{(0.93)^2(100)^2}{m} + \frac{4.6^2}{n_c} + \frac{4.7^2}{n_p}}$$

when V_t = coefficient of variation (standard deviation in per cent of mean),

m = total number of platelets counted,
n_c = number of chambers used,
n_p = number of pipettes used

The first term under the square root represents the *field* error, the second and the third terms the *chamber* and *pipette* errors, respectively.

The field error is due to random distribution of platelets in the chamber. This is the Poisson type distribution and the standard deviation therefore should equal $\sqrt{m}$, or $\frac{S.D.}{\sqrt{m}} = 1$ (m = number of cells counted).

However, using their own method for counting platelets, Brecher & Cronkite (1950) found $\frac{S.D.}{\sqrt{m}} = 0.93$ when a careful study of the field error was undertaken.

In the present work an estimate of the field error in Nygaard's method was made as follows. An arbitrary date was selected from the records, and the number of platelets counted in ten fields of twenty consecutive chambers was tabulated. Each field represented 20 C-squares ($20 \times 1/1{,}000$ c.mm). The standard deviation of the ten readings in each chamber was computed in the usual manner, and the arithmetic mean of $\frac{S.D.}{\sqrt{m}}$ calculated. A value of 0.935 was found, which is in good agreement with the result of Brecher and Cronkite.

The "pipette error" can be neglected if macro-pipettes are used (Berkson *et al.* 1940). Thus, the expected coefficient of variation of platelet counts by Nygaard's method in this work may be calculated from the following formula:

$$V_t = \sqrt{\frac{(0.93)^2(100)^2}{m} + \frac{4.6^2}{n_c}}$$

By use of this formula, the coefficient of variation is calculated as 3.86 per cent if 1,000 platelets are counted in each of two chambers. In this work 10 syringes, the calibration of which differed by no more than ± 1 per cent were used. The motor used for emptying the syringes had speed variations of ± 1 per cent. The "pipette error" would therefore be expected to be small.

The "chamber error" is due mainly to differences in depth among haemocytometers. When, as in this work, only two chambers are used for

one platelet count, it will be a matter of chance whether the "chamber error" is of the same size as indicated by Berkson's formula. In addition, since the two chambers for a single count were taken from a pool of four in no systematic fashion, the "chamber error" may vary from day to day.

An estimate of the precision of the entire procedure, which is included in the estimation of platelet adhesiveness, was obtained in the following manner. Double testing (involving the use of two syringes, two columns and 4 counting chambers) was performed on blood samples from 72 normal persons. The standard deviation and the coefficient of variation of the platelet count before (T_1) and after (T_2) the passage through the column were calculated from these values (see Table 1). Before passage through the column the platelet count coefficient of variation was 3.35 per cent. The random distribution of platelets alone would be expected to give a coefficient of variation of $\sqrt{9.35} = 3.06$ per cent. This indicates that the combined chamber and pipette errors must have been of very little consequence.

Table 1. *Statistics on double tests for platelet adhesiveness on blood samples from 72 healthy individuals*

	Before passage through column	After passage through column
Mean platelet count per c mm	217,500	128,900
Range of platelet counts, per c.mm	138,000—421,000	72,000—275,000
Number of experiments	72	72
S D., platelets per c.mm	7,287	8,507
Coefficient of variation (%)	3 35	6 60

The coefficient of variation after passage through the column was 6.60 per cent. Since this includes a component due to variations in the number of platelets adhering to the beads in the column, such a difference might be expected. This "column error" can be estimated, thus $\sqrt{6.60^2 - 3.35^2} = 5.69$ per cent.

The main interest, however, concerns the precision of the *difference in counts before and after the passage.* The standard deviation of this difference is·

$S.D._{Diff} = \sqrt{S.D.^2_{Before} + S.D.^2_{After}}$, which can be written:

$$\sqrt{\left(\frac{V_{tb} \times T_1}{100}\right)^2 + \left(\frac{V_{ta} \times T_2}{100}\right)^2}$$

where V_{tb} = coefficient of variation of platelet count before passage through the column,

V_{ta} = coefficient of variation of platelet count after passage through the column,

T_1 = platelet count before passage through the column,

T_2 = platelet count after passage through the column.

In the present work double counts of 1,000 cells each were made both before and after the passage (except for blood samples from thrombocytopenic patients). The standard deviation of the number of adhesive platelets is therefore:

$$\frac{1}{\sqrt{2}}\sqrt{\left(\frac{3.35 \times T_1}{100}\right)^2 + \left(\frac{6.60 \times T_2}{100}\right)^2}$$

The 95 per cent confidence interval for the true number of adhesive platelets per c.mm will be:

$$\text{Observed number} \pm \sqrt{2}\sqrt{\left(\frac{3.35 \times T_1}{100}\right)^2 + \left(\frac{6.60 \times T_2}{100}\right)^2}$$

If the mean of the double count before passage is 217,500 and the mean of the double count after passage 128,900 (the means from Table 1), the 95 per cent confidence interval for adhesive platelets is: 88,600 ± 15,800

It may be noted that the "chamber error" could be reduced and the precision thereby enhanced, if one were to use the same chamber before and after the passage. This is, however, technically unpractical and is of no great importance in the present work where the total error is little more than that which can be attributed to the "field error".

A further check on the precision of the method was made with blood samples from 14 thrombocytopenic patients. Here, it was not possible to count more than average 460 cells in each of two chambers before the passage and 315 cells after the passage. The results are given in Table 2. If only the field error is influencing the results, the expected coefficient of variation before passage is approximately $\frac{100}{\sqrt{460}} = 4.7$ per cent which agrees with the observed total error (4.69 per cent). Accordingly, the observations on thrombocytopenic blood confirm the conclusion previously reached, that "chamber error" and "pipette error" are of little importance in this work.

Table 2. *Statistics on double tests for platelet adhesiveness on blood samples from 14 thrombocytopenic patients*

	Before passage through column	After passage through column
Mean platelet count per c mm	35,000	19,000
Range of platelet counts, per c.mm	8,000—60,000	6,000—39,000
Number of experiments	14	14
S D., platelets per c.mm	1,620	1,610
Coefficient of variation (%)	4 69	8 35

After passage, the expected coefficient of variation based on "field error" only, is approximately: $\frac{100}{\sqrt{315}} = 5.6$ per cent. The observed coefficient of variation is 8.4 per cent, which incorporates the effect of the column passage. The component due to this passage ("column error") can be estimated as: $\sqrt{8.4^2 - 5.6^2} = 6.3$ per cent, which compares favourably with the value observed for the same component in healthy individuals, viz 5.69 per cent.

Therefore, the following formula appears applicable at the 95 per cent confidence interval for the true number of adhesive platelets per c.mm if n_b cells are counted in each of two chambers before passage and n_a cells counted in each of two chambers after passage:

(1) Observed number $\pm \sqrt{2}$

$$\sqrt{\left(\frac{1{,}000 \times 3.06^2}{n_b} + 0.29^2\right)\frac{T_1^2}{100^2} + \left(\frac{1{,}000 \times 3.06^2}{n_a} + 0.29^2 + 5.69^2\right)\frac{T_2^2}{100^2}}\ .$$

Summary

A short description of earlier methods for the estimation of platelet adhesiveness is given, and the methodological errors are emphasized. The main errors are:

1 The time of contact between platelets and glass surface is too long (Wright's method).

2 The glass surface area is not standardized (Moolten & Vroman's and Marx & Derlath's methods)

3. The contact time is not standardized (Rovatti's method).

4 The amount of anticoagulant added is not adjusted according to the haematocrit (all methods).

The conditions required for a reliable *in vitro* method for estimation of platelet adhesiveness are discussed, and a new method is described. Nygaard's method for platelet counting was used for reasons discussed. The practical performance of the method is described. Under the chosen conditions, the true value of the number of adhesive platelets is given by formula (1) on p. 26.

3. THE EFFECT OF VARIATIONS IN THE EXPERIMENTAL CONDITIONS

A. *Storage of citrated blood at room temperature*

A decrease in the number of platelets during storage would influence the results. Such a loss could be due, in part, to adherence of the platelets to glass (Baar & Szekely 1929). Theoretically then the ratio adhesive platelets/platelet count might be altered by storage even if platelet adhesiveness were not influenced *per se*. Therefore, studies were conducted to determine the effect of storage.

1 Effect on platelet count

Earlier investigations. Baar & Szekely (1929) studied the decrease in platelet number of citrated blood stored in glass tubes at room temperature. They found great variations with an average decrease of about 30 per cent after storage for 30 minutes.

Table 3 *The platelet count in citrated whole blood 1 minute and 30 minutes after withdrawal*

Sample no	Platelet count, × 1,000, 1 minute after withdrawal of the blood	Platelet count, × 1,000, 30 minutes after withdrawal of the blood
1	213	203
2	168	162
3	178	192
4	184	192
5	262	260
6	236	235
7	184	183
8	217	225
9	206	199
10	207	196
Mean	206	205

The blood was kept in siliconized tubes at room temperature

Personal investigation. Citrated blood from 10 healthy persons was studied. The platelets were counted immediately after withdrawal, and after 30 minutes of storage at room temperature. The results are shown in Table 3. In contrast to the results of Baar & Szekely, no platelet loss was noted under these conditions. A possible explanation is that siliconized needles and glassware were used in the present investigation, whereas Baar & Szekely used untreated needles and tubes. The use of silicone-coated equipment minimizes platelet loss during collection of blood and improves the stability of platelets, factor V and antihaemophilic A factor (factor VIII) during storage, probably by retarding the activation of the coagulation mechanism (Mustard 1957).

2. Effect on platelet adhesiveness

Earlier investigations. Moolten & Vroman (1949 a) found adhesive platelets increased in two persons and decreased in a third after storage of citrated blood in syringes for about one hour. A systematic investigation of this problem has not been reported.

Personal investigation. A sample of blood was taken from each of 10 healthy persons. In addition, blood specimens were drawn from one person on 5 consecutive days. The platelet adhesiveness was determined 5, 10, 15, 20 and 25 minutes after withdrawal of the blood. The results are shown in Table 4.

The adhesiveness increased during the first 15 minutes after withdrawal, followed by a stable period of 10 minutes.

These observations demonstrate the necessity of testing the platelet adhesiveness between 15 and 25 minutes after withdrawal of the blood. In all later experiments, the adhesiveness was measured 15 minutes after withdrawal of the blood.

B. *Variation of temperature*

Previous investigations. Marx & Derlath (1957) found a considerable decrease in platelet adhesiveness when the blood was cooled to 4° C. They assumed that the decrease probably was caused by clumping of the platelets, and, consequently, reduction of the number before the test was performed. It should be mentioned, however, that cooling increases the viscosity and thus reduces the contact area.

Personal investigation Citrated blood was collected from five normal persons. Immediately after mixing with citrate, the blood was transferred

Table 4 *The effect of storage on platelet adhesiven[ess]*

Time after withdrawal of the blood		Sample number 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Mean
Total number of platelets, × 1,000		206	176	150	238	138	178	207	262	236	207	180	207	216	207	207	
Adhesive platelets, %	*5 min*	45	54	56	35	46	33	27	33	26	43	45	29	46	37	43	39.9
Non-adhesive platelets, × 1,000		114	80	66	154	74	120	151	176	174	119	99	148	116	130	119	
Adhesive platelets, %	*10 min*	55	67	55	44	43	41	38	39	39	41	46	34	46	45	41	44 9
Non-adhesive platelets, × 1,000		92	58	67	134	79	105	129	160	144	121	97	136	116	114	121	
Adhesive platelets, %	*15 min*	60	65	60	50	48	41	36	34	34	54	62	37	51	52	54	49.2
Non-adhesive platelets, × 1,000		83	62	60	120	72	105	133	172	157	96	68	131	106	99	96	
Adhesive platelets, %	*20 min*	59	60	59	50	49	42	39	41	34	51	62	43	46	55	51	49 4
Non-adhesive platelets, × 1,000		85	71	62	120	71	104	126	154	156	101	69	118	116	94	101	
Adhesive platelets, %	*25 min*	59	60	57	47	43	49	43	54	34	42	63	46	48	52	42	49.3
Non-adhesive platelets, × 1,000		85	70	64	124	79	91	118	121	157	120	66	111	113	99	120	

Citrated whole blood was kept in siliconized tubes at room temperature After 5, 10, 15, 20 and 25 minutes the platelet adhesiveness was tested. Samples nos. 1—10 were drawn from different persons, nos. 11—15 from one person on 5 different days

to 5 ml syringes, which had been kept for 2 hours at 0°, 20° and 37° C, respectively. The filled syringes were incubated at the respective temperatures for 20 minutes. The platelet adhesiveness was then estimated using glass bead columns at the same temperature as the blood. The results are shown in Table 5.

Table 5. *The effect of temperature on platelet adhesiveness*

Sample no.	Platelet count, × 1,000	Adhesive platelets, × 1,000			Adhesive platelets, %		
		0° C	*20° C*	*37° C*	*0° C*	*20° C*	*37° C*
1	180	89	120	125	50	67	70
2	194	117	113	115	60	58	59
3	201	124	108	116	62	54	57
4	260	78	87	108	30	33	42
5	237	57	63	80	24	26	34
				Mean	45	48	52

The samples were drawn from five healthy persons

The adhesiveness decreased at lower temperature in three instances (nos 1, 4, and 5) and remained constant in two (nos. 2 and 3). The mean values indicate that temperature changes between 0 and 37° C have little, if any, influence on platelet adhesiveness Therefore, slight variations of the room temperature (18-20° C) are without detectable influence on the determinations. Thus, in later experiments, all tests were performed at room temperature

C. *Variation of the contact time*

Previous investigations. By electronmicroscopic examination of parlodion films which were immersed in heparinized blood, De Robertis *et al.* (1953) found that the process of spreading out and adhesion of platelets to the foreign surface took place in "a very short period of 5-30 minutes". Using anoptral contrast microscopy, Kotilainen & Wilska (1955) found that numerous platelets adhered to a glass slide within 20-30 seconds. Wright (1941) found 30 per cent adhered platelets after 20 minutes, 40 per cent after 40 minutes and 60 per cent after 80 minutes of contact with glass.

Solandt & Best (1940) reported that the first platelet thrombi were seen after 6 minutes of extracorporeal circulation through a glass chamber.

In vivo, the platelets immediately adhere to the cut edges of a vessel (Roskam *et al.* 1955).

Personal investigation. The contact time was varied by means of the interchangeable gears of the mechanical device for delivery of the blood from the syringe, and platelet adhesiveness estimated on samples of citrated blood from one healthy person as shown in Table 6. Since it was shown that the number of adhered platelets increased as the contact time was prolonged, the contact time was standardized. In order to record both hypo- and hyperadhesiveness, a contact time of 30 seconds was chosen.

Table 6. *The effect on platelet adhesiveness of increasing contact time with a constant glass contact area (5 grams of beads)*

Duration of contact, Secs.	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
24	175	108	62
		108	62
		122	70
		121	69
		113	65
		118	67
	Mean	115	66
30	197	134	68
		133	68
		132	67
		117	59
		123	63
		114	58
	Mean	125	64
45	204	150	74
		152	74
		153	76
		150	74
		153	75
		154	76
	Mean	152	75
55	199	161	81
		154	78
		149	75
		160	80
		153	77
		163	82
	Mean	157	79

The results are recorded in number and per cent of adhesive platelets.

D. Variation of the area of glass surface

Previous investigations. Marx & Derlath (1957) found 50 per cent adhesive platelets when using a capillary tube with a bore of 1.174 mm. By use of "ganz fein ausgezogene Kapillare" (in which case the glass area is increased in relation to the blood volume) they found 84 per cent adhesive platelets.

Personal investigation. The following tests utilized a constant delivery of blood, since it was found that, if gear changes were made in the mechanical device, tests could not be performed within the 15-25 minutes time limit. This introduces an error, because the time of contact increases with increasing length of the glass bead column. However, earlier experiments (p. 31) showed that this error is small. The adhesiveness of platelets in blood from three normal individuals was tested with 2.5, 5, 7.5, 10, and 15 grams of glass beads. In another series, blood from two additional normal persons was tested with 5, 10, and 20 grams of beads. The results are shown in Table 7. The number of adhesive platelets increased almost in

Table 7. *The effect of increasing glass area surface on platelet adhesiveness*

Sample no.	Platelet count, × 1,000	Gm of glass beads used in the test					
		2.5	5	7.5	10	15	20
1	200	13	32	49	60	80	
2	166	35	44	61	69	85	
3	183	20	32	56	59	70	
4	189		43		75		91
5	421		35		65		93
	Mean	23	37	55	66	78	92

The results are recorded in per cent of adhesive platelets The samples were from five healthy persons.

proportion to the increase of the glass surface area Thus, the mean value of the platelet adhesiveness increased from 37 to 66 per cent when the glass area was increased twice (5 to 10 gm of beads). From Table 6 it can be calculated that an increase of the contact time more than twice (from 24 to 55 seconds) gave only 20 per cent increase of the platelet adhesiveness (per cent of per cent) Variation of the area of the glass surface therefore influenced the platelet adhesiveness to a greater degree than did variation of the contact time. Thus strict standardization of the amount of glass beads is necessary.

E. The influence of different anticoagulants

Previous investigations. Wright (1941) demonstrated a decrease of the adhesiveness of platelets with increasing concentrations of oxalate, heparin, and chlorazol dyes. Hugues (1953), studying normal haemostasis *in vivo*, found that high concentrations of heparin and citrate in the irrigation fluid inhibited the formation of a platelet plug, whereas smaller concentrations had no influence. Lundevall (1958, pp. 37-39) showed that EDTA was more effective in eliminating the platelet-clumping ability of serum than were citrate and oxalate.

Personal investigation

a. Citrate

Nine ml portions of blood from 5 normal persons were collected in siliconized tubes containing 1 ml of 100, 150, 200, and 300 mM solutions of trisodium citrate dihydrate, respectively. After a 15-minute interval the platelet adhesiveness was tested. Table 8 shows that increasing concentrations of citrate produced gradual decrease in platelet adhesiveness. At a final concentration of 30 mM citrate in blood, the platelets were almost non-adhesive. It is therefore important that the citrate concentration be kept at a constant level.

Table 8. *The effect of increasing concentrations of citrate on platelet adhesiveness*

Sample no.	Platelet count, × 1,000	Concentration of citrate in whole blood, mM			
		10	15	20	30
1	197	42	25	19	1
2	220	38	21	15	3
3	299	34	18	14	−2
4	223	32	13	6	4
5	218	28	19	15	5
	Mean	35	19	14	2

The results are recorded in per cent of adhesive platelets. The samples were drawn from five healthy persons.

b. Oxalate

Nine ml portions of blood from 3 normal persons (nos. 1, 4 and 5 in the preceding experiment) were collected in four siliconized tubes containing 1 ml portions of 100, 150, 200, and 300 mM solutions of potassium

oxalate respectively. After an interval of 15 minutes, the platelet adhesiveness was tested. The total count in the different samples showes slight increase with increasing concentrations of oxalate (mean value at 10 mM 229,000 v. 262,000 at 30 mM), whereas the total count remained constant in the series with increasing concentrations of citrate. This indicates that platelets are more adhesive in oxalated blood than in citrated blood. A tendency of the platelets to form clumps in the 10 and 15 mM oxalate solutions was noticed. The results are shown in Table 9. In this table the total count of platelets is the value found in the blood sample with the highest concentration of oxalate. The calculation of the adhesive platelets, therefore, also includes the platelets lost by adhesion to the wall of the collecting tube.

Table 9. *The effect of increasing concentrations of oxalate on platelet adhesiveness*

Sample no.	Platelet count, × 1,000	Concentration of potassium oxalate in whole blood, mM			
		10	15	20	30
1	233	79	72	62	26
2	249	70	62	47	30
3	305	76	65	54	24
	Mean	75	66	54	27

The results are recorded in per cent of adhesive platelets The samples were drawn from three healthy persons

The experiment gave two valuable items of information. First, platelet adhesiveness decreases with increasing oxalate concentration; second, platelet adhesiveness in oxalated blood is much higher than in equimolar concentrations of citrate. In addition, a falsely low platelet count was found at low concentrations of oxalate. At these concentrations, platelet clumps were also found. Oxalate, therefore, was rejected as a suitable anticoagulant for the present work Furthermore, it should also be avoided as the anticoagulant in all platelet work.

c. EDTA

Previous investigations. EDTA is a highly effective calcium binding agent, especially in neutral and alkaline solutions (Flaschka 1955). It is an effective anticoagulant, but in addition destroys some of the coagulation factors (Leikin & Bessman 1956) EDTA prevents morphological changes and unspecific clumping of platelets. Therefore, it has been recommended

as the anticoagulant for separation of platelets for transfusion (Sprague *et al.* 1952, Aas & Gardner 1958), for morphological platelet studies (Feissly *et al.* 1957); and for serological studies on platelets (Lundevall 1958). Marx & Derlath (1957) found that platelet adhesiveness was 50 per cent lower in blood containing 5.37 mM EDTA than in blood with 20 mM citrate.

Personal investigation. A stock solution of EDTA in saline was made, the pH was adjusted to 7.4 with N 1 NaOH. From this solution four dilutions were prepared. The concentration of EDTA in these was 10, 20, 30, and 40 mM, respectively. One volume of each of these solutions was added to 9 volumes of blood from 3 normal donors. After a 15-minute interval, the adhesiveness of the platelets was measured. The results are presented in Table 10.

Table 10. *The effect of increasing concentrations of EDTA on platelet adhesiveness*

Sample no	Platelet count, × 1,000	Concentration of EDTA in whole blood, mM			
		1	2	3	4
1	233	—*	21	10	—1
2	230	84**	26	17	1
3	248	66**	25	17	6
	Mean	75	24	15	2

The results are recorded in per cent of adhesive platelets. The samples were drawn from three healthy persons.
* Blood clotted ** Blood partially clotted.

The adhesiveness of the platelets could not be estimated in blood where the concentration of EDTA was less than 2 mM, due to partial coagulation. At final EDTA-concentrations above 4 mM, the adhesiveness of the platelets was zero. At equimolar concentrations, EDTA is much more effective than citrate for prevention of platelet adhesion. The range of EDTA concentrations that could be used for studies on platelet adhesiveness is too narrow for the present method.

d. Heparin

Previous investigations Copley & Robb (1942 a) found a pronounced decrease in the platelet count when blood was incubated with heparin *in vitro*, but no effect on the platelet count in mice treated with heparin. Treatment of dogs with heparin, however, gave a transient fall of the number of platelets (Copley & Robb 1942 b). Fidlar & Jaques (1948)

found a decrease in the platelet number after injections of heparin in dogs and humans. Quick *et al.* (1948) found the same in dogs, but not in human beings. After injection of heparin in hamsters, Copley (1948) demonstrated platelet emboli in the vessels of the cheek pouch. The unspecific clumping of platelets is not prevented by heparin (Lundevall 1958, p. 45). Wright (1941) found a pronounced decrease in platelet adhesiveness with increasing heparin concentrations *in vitro*.

Personal investigation. Ten ml portions of blood from 3 normal persons were mixed with 2, 4, and 8 mg of heparin. The platelet adhesiveness was tested after 15 minutes.

The results were confusing. Both the total count and the calculated number of adhesive platelets showed great variations without any systematic trend for different concentrations of heparin. This was due to difficulties in counting, partly because the platelets, in all samples, formed clumps in the counting chambers, and possibly because platelets adhered to the wall of the glass tube before the actual test of adhesiveness. The number of non-adhesive platelets, however, showed a constant increase in all samples with increasing concentration of heparin. This is illustrated in Table 11. The finding of an increased number of non-adhesive platelets with increasing concentrations of heparin, indicates that the platelet adhesiveness decreased with increasing concentration of heparin.

Table 11. *The effect of increasing concentrations of heparin on platelet adhesiveness*

Sample no.	Concentration of heparin (mg/ml blood)					
	0 2		0 4		0 8	
	Platelet count, × 1,000	Non-adhesive platelets, × 1,000	Platelet count, × 1,000	Non-adhesive platelets, × 1,000	Platelet count, × 1,000	Non-adhesive platelets, × 1,000
1	—	30	—	49	153	62
2	150	62	138	79	230	114
3	235	99	256	112	210	176
Mean		64		80		117

In this table the number of *non-adhesive* platelets is recorded for reasons discussed in the text
The samples were drawn from three healthy persons

In conclusion, heparin added to blood was found to produce a high degree of platelet clumping. The total count of platelets fluctuated without systematic trend with varying heparin concentrations. Heparin therefore could not be used for estimation of platelet adhesiveness by the present method.

F. The effect of haematocrit variations

Previous investigations To my knowledge, studies on the influence of the haematocrit value on platelet adhesiveness have not been reported.

Personal investigation. In *citrated plasma* from 60 donors the average value of the adhesiveness of platelets was 4 per cent, whereas the average value in *citrated blood* was 42 per cent (pp. 61 & 59). This difference indicates that the presence of red cells has a decisive influence on the adhesiveness of the platelets This conclusion was confirmed by the following experiments.

a) Nine volumes of blood from a normal person were drawn by strict silicone technique into a siliconized tube containing 1 volume of 3.13 per cent sodium citrate solution. The tube was centrifuged at 190 G for 15 minutes. The platelet-rich plasma was pipetted off. The "buffy coat" and the upper layer of the red cells were removed and discarded. The remaining red cells were centrifuged for 5 minutes at 900 G, and the supernatant plasma pipetted off and discarded By mixing various proportions of the packed red cells and the platelet-rich plasma thus obtained, whole blood samples with different haematocrit values, but with a constant citrate concentration, were available for testing. The adhesiveness of the platelets was estimated in all samples Table 12 shows that the adhesiveness was closely correlated with the haematocrit value

Table 12. *The effect of increasing haematocrit on the platelet adhesiveness*

Sample no	Haematocrit, volume %	Platelet count, × 1,000	Adhesive platelets, %
	0	351	2
1	20	247	21
	50	52	35
	80	72	43
	0	297	5
2	20	220	25
	50	135	46
	80	69	70
	0	461	1
3	20	318	10
	50	216	41
	80	124	60
	0		3
	20		19
Mean	50		41
	80		58

The different haematocrit values were obtained by mixing packed red cells and platelet-rich plasma in various proportions The blood was drawn from three healthy persons

In this experiment, the true haematocrit value is somewhat lower than the calculated value, due to some plasma contamination of the packed red cells. Therefore, the investigation was repeated in another way. Two samples of citrated blood from one donor were centrifuged for 15 minutes at 190 G. From one of these tubes, a certain amount of platelet-rich plasma was removed. After mixing the red cells and the remaining platelet-rich plasma, the tube contained citrated whole blood with a high haematocrit value. Platelet-rich plasma pipetted from the second tube was mixed, in different proportions, with citrated whole blood from the same donor. Thus blood samples with a haematocrit value lower than the normal were produced.

Table 13. *The effect of varying the haematocrit on the platelet adhesiveness*

Sample no.	Original haematocrit	Haematocrit of mixture	Platelet count, × 1,000	Adhesive platelets, %
		21	216	15
		29	221	25
1	41.5	31	225	30
		37	237	35
		47	224	51
		0	347	0
		16	237	8
2	45.0	32	205	28
		41	182	49
		58	174	55
		0	431	6
		15	315	11
3	42 5	31	248	26
		38	260	46
		55	182	59
		0	338	8
		16	235	5
4	45 0	32	208	33
		41	197	37
		58	132	52
		0	238	—3
		15	196	16
5	42.0	30	164	32
		38	162	38
		54	123	60

The different haematocrit values were obtained by platelet-rich plasma addition to, or removal from, citrated blood.

A total of five samples from different donors was prepared in this manner, and the platelet adhesiveness determined in each. Table 13 shows that there was a close correlation between the adhesiveness of the platelets and the haematocrit value. In this experiment, the curve was steeper than in the preceding one (Table 12). This was expected since the true haematocrit values in the first experiment were lower than calculated.

In these experiments *a close correlation* between the adhesiveness of the platelets and the haematocrit value in the blood was demonstrated. With a contact time of 30 seconds and a glass surface of 5 grams of glass beads (standard conditions in the method), the adhesiveness of the platelets, expressed in per cent, was approximately equal to the haematocrit value.

When platelet adhesiveness is estimated in blood from patients with abnormal haematocrit values, correction must be made for the observed number of adhesive platelets (observed number of adhesive platelets $\times \frac{45}{\text{observed haematocrit}}$) As described above, it is also possible to adjust the haematocrit value prior to the estimation and record the platelet adhesiveness in per cent. This is the correct way if the purpose is to investigate the property of platelets to adhere *in vitro*. However, it is possible that the assay without correction for the observed number at abnormal haematocrit, or without adjustment of the haematocrit prior to the estimation, reflects more closely the property of the platelets of adhering *in vivo* (the haemostatic effect of the platelets) This point will be discussed later (p. 94).

G. *The effect of untreated and siliconized equipment*

Previous investigations Moolten *et al* (1949 b) demonstrated that intact endothelium was non-wettable, whereas injured endothelium became wettable. In vessels with intact endothelium the blood remained fluid for a long time after the death of the animal, and the platelets did not adhere to the walls. Jaques *et al.* (1946) demonstrated that no coagulation occurred for hours, and the number of platelets remained stable for half an hour if platelet-rich native plasma was stored in siliconized tubes

Favre-Gilly *et al.* (1950) and Sprague *et al.* (1952) found that the effect of platelet transfusions was improved when siliconized equipment was used The use of silicone-coated or plastic equipment in blood collection has been found to minimize platelet loss (Muether *et al.* 1953, Mustard & Walker 1957) These reports have led to the opinion that platelets do not adhere to silicone-coated surfaces. Moolten & Vroman (1949 a) dem-

In this way, three samples of blood were prepared, with a constant haematocrit, but with different numbers of platelets. The adhesiveness of the platelets in each sample was measured. The experiment was repeated with blood from another donor. Table 16 shows that the adhesiveness expressed in per cent was constant and independent of the number of platelets in the blood sample.

Table 16. *Platelet adhesiveness at different platelet counts*

Sample no.	Packed red cells, ml	Platelet-rich plasma, ml	Platelet-poor plasma, ml	Platelet count in mixture, × 1,000	Adhesive platelets, %
1	2.5	2.5	0.0	114	44
	2.5	1.0	1.5	64	46
	2.5	0.5	2.0	57	45
2	2.5	2.5	0.0	216	47
	2.5	1.0	1.5	108	51
	2.5	0.0	2.5	31	48

The platelet count was varied by mixing packed red cells, platelet-rich, and platelet-poor plasma from the same sample of citrated blood

In conclusion: Investigation reveals that a correct picture of platelet adhesiveness may be obtained in blood samples from thrombocytopenic patients if the adhesiveness is expressed in per cent

I. The effect of meals

Previous investigations. Cullen & Swank (1954) reported that after fatty meals there was an inconstant aggregation of platelets in the vessels of the cheek pouch in hamsters Loughry & Cole (1954) found a 40-50 per cent decrease in the number of circulating platelets after intravenous administration of fat emulsions in humans. Meng *et al.* (1956) found a similar decrease in dogs after intravenous administration of fat emulsions, but not after fatty meals. These observations indicate a possible effect of fat on platelet adhesiveness. No studies *in vitro* on this subject have been reported.

Personal investigation. The effect of ordinary meals on the adhesiveness of the platelets was investigated for the purpose of standardization of the method, but the effect of extremely fatty was not studied.

Blood from 5 normal persons was re, and one and three hours after a breakfast onsisting milk, bread, butter,

cheese, and one egg In three cases (persons nos. 3–5) 150 ml of cream were added to the meal. The platelet adhesiveness was tested and the results are shown in Table 17.

Table 17. *The effect of a meal on platelet adhesiveness*

Sample no	Time between meal and withdrawal	Platelet count, × 1,000	Adhesive platelets, %
1	Before meal	195	45
	1 hour after	200	50
	3 hours after	218	49
2	Before meal	216	44
	1 hour after	237	46
	3 hours after	228	43
3	Before meal	194	45
	1 hour after	208	52
	3 hours after	201	41
4	Before meal	207	37
	1 hour after	224	38
	3 hours after	229	41
5	Before meal	291	51
	1 hour after	266	46
	3 hours after	279	53
Mean	Before meal		44
	1 hour after		46
	3 hours after		45

The meal contained about 30 gm fat (samples 1—2) or about 100 gm fat (samples 3—5).

A very slight increase both in the platelet count and in the percentage of adhesive platelets was found one hour after the meal in all persons except for one (person no 5). The result shows that in estimation of platelet adhesiveness no great error results from sampling blood before or after an ordinary meal In the present work, all blood samples were drawn one or two hours after breakfast.

J The effect of increased osmolarity

Previous investigations Lundevall (1958) found a decrease of the platelet clumping in strongly hypertonic solutions

Personal investigation. Increased osmolarity of plasma was obtained by addition of glucose to citrated blood A 1,000 mM solution of glucose

in saline was prepared. To 4.5 ml of citrated blood in each of three tube 0.125, 0.25, and 0.5 ml respectively of this solution was added. A fourth tube, containing blood without glucose, served as a control. Subsequently 0.9 per cent saline was added to give a final volume of 5 ml in each tube In this way, the four blood samples had a constant ionic strength and were diluted to the same degree, while the osmolarity in plasma was varied. The platelet adhesiveness was then tested. The experiment was repeated with blood from 4 additional donors. The results are shown in Table 18. The results were confusing with regard to the percentage of adhesive platelets This was due to variations in total platelet count of the mixtures with

Table 18. *The effect of increasing osmolarity on the platelet adhesiveness*

Sample no.	Concentration of added glucose in whole blood, mM	Platelet count, × 1,000	Non-adhesive platelets, × 1,000	Adhesive platelets, %
	0	159	123	22
1	25	162	115	29
	50	169	119	29
	100	179	145	19
	0	275	184	33
2	25	268	190	29
	50	267	212	21
	100	316	240	24
	0	165	84	49
3	25	153	102	33
	50	155	110	29
	100	152	132	14
	0	168	101	40
4	25	174	107	39
	50	160	113	29
	100	165	138	16
	0	237	89	62
5	25	243	93	62
	50	243	123	50
	100	263	218	17
	0			41
Mean	25			38
	50			32
	100			18

The osmolarity was increased by adding different amounts of glucose to the citrated blood.

different osmolarity. When the number of non-adhesive platelets was recorded, the interpretation was simpler. The number of non-adhesive platelets increased in all samples as the glucose concentration was increased beyond 50 mM. When the increase was less than 50 mM, the number of non-adhesive platelets remained almost constant. Therefore, variations in glucose concentration within the physiological range did not influence the adhesiveness of the platelets. When greater amounts of glucose were added, the platelet adhesiveness decreased.

K. *The effect of varying both the ionic strength and the osmolarity*

Previous investigations. Lundevall (1958) found a decrease in platelet clumping at increasing ionic strength and osmolarity.

Table 19. *The influence of ionic strength (and molarity) on the platelet adhesiveness*

Sample no.	Relative ionic strength,* %	Platelet count, × 1,000	Adhesive platelets, %
1	90	143	48
	100	146	53
	120	157	18
2	90	152	20
	100	159	37
	120	156	20
3	90	155	42
	100	154	50
	120	138	20
4	90	189	41
	100	193	43
	120	186	23
5	90	199	38
	100	189	36
	120	190	22
Mean	90		38
	100		44
	120		21

* 90% relative ionic strength 10 ml of citrated blood + 2.5 ml 0.45% NaCl.
100% relative ionic strength 10 ml of citrated blood + 2.5 ml 0.9% NaCl.
120% relative ionic strength 10 ml of citrated blood + 2.5 ml 1.8% NaCl.

Citrated blood was drawn from five healthy persons.

Personal investigation. Addition of hyper- or hypotonic NaCl solutions to citrated blood causes changes in both ionic strength and osmolarity. This combined effect of NaCl solutions on platelet adhesiveness was tested in the following experiment.

From each of five donors, three samples of 10 ml citrated blood were collected. To each, 2.5 ml portions of 0.45, 0.9, and 1.8 per cent NaCl solutions were added respectively. The platelet adhesiveness in these samples was measured and the results are shown in Table 19. Platelet adhesiveness diminished both with decreased and increased osmolarity and ionic strength of the blood, but minor fluctuations, such as may occur *in vivo*, did not influence the platelet adhesiveness. In experimental work, however, the osmotic pressure and ionic strength of the blood must be kept constant.

L. The effect of Triton WR-1339

Previous investigations. Triton WR-1339 is a surface-active alkyl-aryl polyether alcohol which prevents platelet clumping and facilitates the resuspension of platelets after centrifugation (Lundevall 1958). Kotilainen & Wilska (1955) found that a similar product, Tween 80, prevented the adhesion of platelets to "foreign surfaces". Minor & Burnett (1953), Aas & Gardner (1958), and many other authors added Triton WR-1339 to blood samples for the collection and separation of platelets.

Personal investigations. A 1 per cent solution of Triton WR-1339 in saline was prepared. To 4.5 ml portions of citrated blood from five normal persons 0.5 and 1 ml of the Triton solution were added respectively. Samples of blood to which no Triton was added served as controls The final volume in each tube was adjusted to 5.5 ml with physiological saline. The platelet adhesiveness was estimated after a 15-minute interval, and the results are shown in Table 20.

The platelet adhesiveness decreased about 50 per cent at a concentration of 0.09 per cent Triton, but no further decrease was observed when the concentration was increased to 0.18 per cent. These results confirm the observations of earlier authors.

Summary

The experiments performed to develop a standardized method for the assay of platelet adhesiveness are described The results may be summarized as follows:

The blood samples should be tested in the interval between 15 and 25 minutes after collection since the platelet adhesiveness is constant within this interval.

Table 20. *The effect of Triton WR-1339 on platelet adhesiveness*

Sample no.	Concentration of Triton in citrated blood, %	Platelet count, × 1,000	Adhesive platelets, %
1	0.00	142	29
	0.09	142	17
	0.18	146	17
2	0.00	137	30
	0.09	132	14
	0.18	129	14
3	0.00	146	24
	0.09	145	7
	0.18	162	12
4	0.00	125	32
	0.09	119	20
	0.18	124	17
5	0.00	164	43
	0.09	166	19
	0.18	178	19
Mean	0.00		32
	0.09		15
	0.18		16

The contact time between the glass surface and the blood should be 30 seconds. Slight deviations from this standardized time period are of minor importance.

The test may be carried out at room temperature. Temperature variations between 0° and 37° C do not significantly alter the results.

The glass surface area is critical. Five grams of glass beads with a diameter of 0.5 mm should be used.

Sodium citrate dihydrate (3.13 g / 100 ml) should be used as anticoagulant. The concentration of citrate in plasma is important, and it must be kept constant. Other anticoagulants are not satisfactory for the reasons stated.

A close correlation between the adhesiveness of the platelets and the haematocrit value in the blood samples is demonstrated.

Collection of blood in uncoated or silicone-coated tubes does not affect the platelet adhesiveness. Silicone-coated cannulas, syringes, and tubes are

used, however, to avoid any possible activation of coagulation factors which may provoke platelet clumping The glass beads used are *not silicone-coated*, as platelets adhere to uncoated glass beads to a greater extent than to siliconized ones.

The percentage of adhesive platelets is independent of the number of platelets in the blood sample. Therefore, the determination may be performed irrespective of the total platelet count.

A light meal does not change the adhesiveness of the platelets. Thus, for the estimation, it is unnecessary to draw the blood from fasting subjects.

Great variations in the osmolarity and ionic strength of the plasma milieu reduce the platelet adhesiveness. Lesser variations, as may occur *in vivo*, do not affect the adhesiveness

The presence of 0 09 per cent or more Triton WR-1339, reduces the adhesiveness to about half the original value.

4. INVESTIGATIONS ON THE PLATELET ADHESIVENESS IN CITRATED PLASMA

A. *The effect of storage at 4° C*

Earlier investigations. Lundevall (1958, p. 27) found that platelets in citrated plasma formed clumps more easily when they were stored in the cold. Zucker & Borelli (1954) found that 85 per cent of the platelets maintained a discoid shape when incubated in their own citrated plasma for 30 minutes at 37° C. After storage at 4° C for 10 minutes, 95 per cent of the platelets became spherical These morphological alterations were reversed by reincubation of the platelet-rich plasma at 37° C In plasma containing EDTA, the platelets were spherical at both 4° and 37° C. Platelets which have adhered to a glass surface (spread-out forms) show changed morphology on incubation at 2-3° C. Their hyaloplasm retracts, leaving long, fine pseudopodia which adhere to the glass. By reincubation at 37° C, some, but not all, of the cells return to their initial state (Bessis 1956, p 463).

These observations indicate that both the morphology and the adhesiveness of platelets change at low temperature, but no efforts have been made to measure the influence of low temperatures on the latter.

Personal investigation. Citrated blood was obtained from three donors and platelet-rich plasma prepared by centrifugation for 15 minutes at *190 G The plasma was divided into 10 ml portions which were stored at*

Table 21. *Storage of citrated platelet-rich plasma at 4° C. effect on platelet count and adhesiveness*

Sample no	Duration of storage	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
1	0 days	478	48	10
	2 "	372	253	68
	3 "	213	130	61
	7 "	82	7	9
2	0 days	462	14	3
	1 "	425	210	50
	2 "	403	189	47
	3 "	317	109	34
	5 "	287	97	34
	8 "	159	18	11
3	0 hours	416	0	0
	3 "	420	92	22
	5 "	393	157	40
	10 "	188	101	54

4° C. After different incubation periods, the platelet adhesiveness of the samples was estimated. The results are shown in Table 21. Two distinct observations were made. First, with storage, a progressive decrease in platelet numbers was observed Second, a marked increase in platelet adhesiveness occurred during the first 24 hours, which was followed by a progressive decrease to the original level in 7-8 days

This surprising finding that adhesiveness is increased greatly during the first 24 hours of storage is important, since it is commonly recommended that temperatures be kept low during many platelet procedures. From the lower part of the table it is seen that the major increase in platelet adhesiveness on storage at 4° C occurred during the initial 3-5 hours

B. *Storage at 4° C followed by storage at 20° C*

Plasma from person no 1 in Table 21 with a platelet count of 213,000, and with 61 per cent adhesive platelets after 72 hours of storage at 4° C, was stored at 20° C for an additional 18-hour period The adhesiveness of the platelets was then estimated Table 22 shows that the platelets again lost their adhesive property This result might be due to a reversibility comparable to the reversibility of the morphological alterations described by Zucker & Borelli (1954) and Bessis (1956) To test this theory an

Table 22. *Platelet count and adhesiveness in citrated platelet-rich plasma stored at 4 and 20° C*

Sample no.	Storage, in hours, at given temp	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
1	72 at 4° C	213	130	61
	72 at 4° C + 18 at 20° C	176	10	6
2	48 at 20° C	518	16	3
	48 at 20° C + 24 at 4° C	472	3	<1

additional, freshly obtained, sample of platelet-rich plasma from the same person (no. 1, Table 21) was stored first for 48 hours at 20° C, and then at 4° C for 24 hours. The results of this experiment are shown in the lower part of Table 22.

After 48 hours of storage at 20° C the adhesiveness of the platelets was only 3 per cent. The adhesiveness was not increased by the subsequent storage at 4° C for 24 hours. Thus it may be concluded that the incubation of platelets in citrated plasma at room temperature, destroys the ability of platelets to adhere to foreign surfaces.

C. *Storage at 20° C and 37° C*

Several samples of platelet-rich plasma from four persons were stored at 20° C and the platelet adhesiveness tested at different intervals. In addition, platelet-rich plasma from one person was stored at 37° C and the adhesiveness tested at short intervals. Table 23 shows the results. Only a slight increase in the adhesiveness was found after six hours of storage at 20° C, whereas no increase was found after 3 hours of storage at 37° C. These findings indicate that the increased adhesiveness in the Table 21 is due to the effect of the low temperature and not to the storage *per se*.

D. *Is the cold-induced increase in adhesiveness the result of alterations of plasma factors or of platelet properties?*

Waaler (1958) found that certain coagulation factors were activated when plasma was stored for 5-7 hours in uncoated glass tubes at 4° C, whereas no activation could be demonstrated after storage at room tem-

Table 23 *Platelet count and adhesiveness in citrated platelet-rich plasma stored at 20 and 37° C*

Sample no.	Time of storage, hours	Temperature of storage, °C	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
1	0	20	478	48	10
	24		480	18	4
	48		518	16	3
	72		523	−9	−2
2	0	20	462	14	3
	24		499	−27	−5
	48		469	7	1
3	0	20	312	24	8
	1½		313	32	10
	3		303	51	17
	6		272	75	27
4	0	20	675	−13	−2
	1		656	34	5
	3		631	34	7
	6		659	120	18
5	0	37	324	25	8
	¾		314	−3	−1
	1¾		310	26	8
	3		294	15	5

perature His "activation product" produced a rapid clumping and viscous metamorphosis of platelets in native plasma However, Waaler (1959) did not find any activation of coagulation factors when the plasma was stored in siliconized tubes, nor did the "activation product" act on platelets in citrated plasma without the addition of calcium In the present experiments, siliconized tubes were used throughout, and the adhesiveness was measured in citrated plasma These observations preclude any relationship between the mechanism of increased adhesiveness and the activation of coagulation factors by storage However, the possibility that other plasma factors may be responsible for the increase noted in platelet adhesiveness is not excluded.

For further investigation of this point, the following experiment was made Platelet-rich plasma from a normal person was stored at 4° C for 48 hours, and the adhesiveness was tested The total number of platelets was 403,000, the number of adhesive platelets 189,000, and the percentage of adhesive platelets was 47. A sample of this plasma was centrifuged for

30 minutes at 2,300 G, and the supernatant, platelet-poor plasma was pipetted off. A sample of fresh platelet-rich plasma from the same donor was prepared. After mixing equal volumes of the platelet-poor and the freshly prepared platelet-rich plasmas the platelet adhesiveness in the mixture was estimated. The mixture contained 299,000 platelets per c.mm, the number of adhesive platelets was - 8,000, and the percentage of adhesive platelets was - 2.

This shows that the increase in platelet adhesiveness at low temperatures is not caused by plasma factors.

E. The influence of EDTA and citrate on the cold-induced platelet adhesiveness

The following experiment was performed in order to find whether the increased adhesiveness at low temperatures was influenced by EDTA and citrate in the same way as these substances affected the adhesiveness of platelets in whole blood (pp 33-35).

Three 4.5 ml samples of platelet-rich plasma from one donor were stored at 4° C for 24 hours. To one portion EDTA was added to a final concentration of 6 mM; to the next portion citrate was added to a final

Table 24 *The effect on cold-induced platelet adhesiveness of EDTA and citrate or increased concentration of citrate*

Sample no.	Concentration in plasma, mM		Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
	EDTA	Citrate			
1	0 6 0	16 16 46	198 296 213	114 —9 42	58 —3 19
2	0 6 0	16 16 46	483 495 528	208 4 107	43 1 20
3	0 6 0	16 16 46	239 232 202	136 27 72	58 12 36
Mean	0 6 0	16 16 46			53 3 25

Platelet-rich plasma was tested after storage for 24 hours at 4° C

concentration of 46 mM; and to the third portion saline was added. In each case the volume addition was 0.5 ml The platelet adhesiveness was measured in each sample. This was repeated with plasma samples from two other persons, and the results are shown in Table 24. The platelet adhesiveness induced by low temperature decreased to about zero in the presence of EDTA, as did the adhesiveness in whole blood (p. 34). By increasing the plasma concentration of citrate from 16 to 46 mM, the cold-induced adhesiveness decreased from about 50 to 25 per cent. In whole blood with a citrate concentration of 10 mM (plasmaconcentration of about 18 mM) the average percentage of adhesive platelets was 35. By increasing the concentration of citrate in whole blood to 25 mM (plasma concentration of about 46 mM) the average percentage of adhesive platelets may be calculated to be about 8 per cent (Table 8) Therefore, the influence of increasing concentrations of citrate on adhesiveness induced by cold was less than on the adhesiveness in whole blood. This observation indicates that these two phenomena are closely related, but probably not identical.

Thus, the platelet adhesiveness in plasma should be estimated in freshly prepared citrated plasma This plasma should not be stored at 4° C for more than approximately 1 hour On the other hand, irreversible damages occur if the platelets are stored at room temperature for 24-48 hours or more. This should be borne in mind in all platelet work, especially in platelet transfusions, since it is reasonable to assume that platelet adhesiveness in some way is related to platelet "viability" (see also Table 34).

Summary

It is demonstrated that the platelets in freshly drawn plasma have nearly no ability to adhere to foreign surfaces When stored at 4° C for a few hours, the platelet adhesiveness increases considerably. This is shown to be due to alteration of the platelets and not to any plasma factor. When stored at room temperature for at least 48 hours, the platelets are probably damaged and the property of adhesiveness is irreversibly lost. This is an important consideration in platelet work.

5. THE PLATELET ADHESIVENESS IN PHYSIOLOGICAL SALINE AND IN ISOTONIC SOLUTION OF CITRATE

Previous investigations. Marx & Derlath (1957) found 34 per cent adhesive platelets in an isotonic citrate-saline solution when the platelets had been washed three times in this solution Bounameaux (1955 a) found

about 35 per cent adhesive platelets in physiological saline when the platelets had not been washed, and the adhesiveness decreased progressively with the number of washings. After washing six times in saline, the adhesiveness was lost. If the platelets were washed with saline-citrate solution, the adhesiveness was lost after the third washing.

Personal investigation. Citrated platelet-rich plasma was prepared from four persons. Each sample was divided into three 5 ml samples, and centrifuged for 20 minutes at 2,300 G. The platelet-poor plasma was pipetted off, and the platelets resuspended respectively in 5 ml of physiological saline, isotonic solution of citrate, and physiological saline containing EDTA (final concentration 10 mM). The resuspension of the platelets was difficult, and in spite of the fact that the different test tubes were handled in a similar manner, varying amounts of platelets were lost during the procedure. This was due to both platelet clumping and adherence of platelets to the wall of the tubes. The result of the estimation of platelet adhesiveness in all tubes is shown in Table 25. The platelet count differed considerably in all tubes. As varying numbers of platelets were lost before the actual tests were performed, the recorded number of adhesive platelets is

Table 25. *The adhesiveness of platelets suspended in saline, in isotonic citrate, and in saline containing 10 mM EDTA*

Sample no.	Platelets suspended in	Platelet count × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
1	Saline	115	21	19
	Citrate	355	78	22
	Saline-EDTA	280	47	17
2	Saline	501	117	23
	Citrate	458	34	8
	Saline-EDTA	362	133	37
3	Saline	125	37	30
	Citrate	[illegible]	21	[illegible]
	Saline-EDTA	[illegible]	[illegible]	[illegible]

only relative. The average value of the platelet adhesiveness was 25 per cent in saline, 10 per cent in the citrate solution, and 26 per cent in EDTA-saline solution. This study permits the following conclusions: Platelets in saline are adhesive; in an isotonic solution of citrate, the adhesiveness is probably diminished; and a 10 mM concentration of EDTA does not abolish the platelet adhesiveness as it does both in whole blood and in citrated plasma stored at 4° C. The platelet adhesiveness found in saline, therefore, is neither identical to "the natural" adhesiveness in whole blood, nor is it identical to the cold-induced adhesiveness in plasma. The most reasonable explanation is that the platelet adhesiveness in saline is an artifact, caused by a denaturation of the surface of the platelets.

6. THE PLATELET ADHESIVENESS IN CITRATED PLASMA CONTAINING DIFFERENT AMOUNTS OF PHYSIOLOGICAL SALINE

The previous experiments indicated that plasma prevents the platelets from undergoing surface alterations. Since saline is often added to platelet-rich plasma in experimental and practical procedures, it is of interest to know if the presence of small amounts of saline in plasma changes the adhesiveness. In the following experiment different amounts of saline were added to platelet-rich plasma, and the adhesiveness was measured 15 minutes later. Table 26 shows that the platelet adhesiveness did not increase significantly upon addition of up to 80 per cent of the total volume of saline.

Table 26 *The platelet adhesiveness in plasma-saline mixtures*

Concentration of saline in mixture, %	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
0	468	6	1
20	379	29	8
50	264	18	7
80	101	11	11

Discussion

The influence of EDTA on the adhesiveness of platelets in citrated blood and in physiological saline shows that platelet adhesiveness in saline is different from platelet adhesiveness in their natural milieu. Assuming that platelet adhesiveness in plasma is related to "viability", the findings

presented indicate that platelets should not be resuspended in saline. The dilution of plasma with some saline, however, does not influence platelet adhesiveness. These facts, as well as the influence of storage at 4° C (pp. 48-50) should be borne in mind when platelets are investigated or resuspended before platelet transfusions. Thus it is concluded that platelets should be kept in their natural milieu (plasma), otherwise artifacts are introduced in investigation and "viability" influenced in transfusion.

Summary

Isolated platelets suspended in physiological saline have an increased adhesiveness as compared to that in plasma. The adhesiveness in saline is probably due to alterations of the platelets (denaturation?). The adhesiveness of the platelets in an isotonic solution of citrate is less than in normal saline. The adhesiveness of the platelets in saline under varying conditions probably does not reflect variations in their natural adhesiveness.

Platelet adhesiveness in healthy persons

The platelet count differs considerably in normal subjects. Sloan (1951) found a mean platelet count of 241,000 with a range of 140,000 - 340,000 in 80 healthy young adults. There was no significant variation in the platelet count of a person during one day and on different days, and no difference between males and females. Brecher *et al.* (1953) confirmed these findings.

Since the total platelet count normally shows individual variations, the *number of adhesive platelets also might be expected to vary. This was* studied in the following experiments

1. THE MEAN VALUE AND THE NORMAL RANGE OF NUMBER AND PERCENTAGE OF ADHESIVE PLATELETS IN CITRATED WHOLE BLOOD FROM HEALTHY PERSONS

Previous investigations In whole blood, containing 0 2 mg heparin per ml, Wright (1946) found an average of 64 per cent adhesive platelets after rotation for 80 minutes, with a range of 58-69 per cent. By the same method, Weiner *et al.* (1948) found a normal range, in oxalated blood, of 60 to 75 per cent after 80 minutes of rotation. Moolten & Vroman (1949 a) reported a range of the "adhesive index" between 1.0 and 1.4 (adhesive index $= \frac{\text{Total platelet count}}{\text{No of non-adhesive platelets}} - 0.3$), corresponding to 60,000 - 110,000 adhesive platelets per c mm. By the same method, Eisen *et al.* (1951) found a mean of 67,000 adhesive platelets with a standard deviation of 18,000 in 100 normal persons Nathanson & Savitsky (1952) found a normal range of the adhesive index between 0.90 and 1.25, while Bobek & Čepelák (1958) found a normal range of 1.04-1.50.

Personal investigation. 104 determinations were performed on citrated whole blood from 60 normal persons ranging in age from 12 to 60 years.

Sixty-three of these samples were from males and 41 from females The total platelet count ranged from 138,000 to 421,000 with a mean of 215,000 per c.mm as shown in Fig. 4.

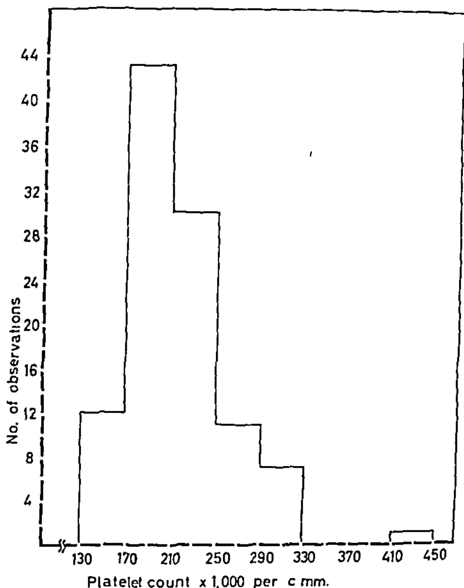


Fig. 4 *Platelet count in 104 samples from 60 healthy persons*
The abscissa shows the platelet count in citrated venous blood (1 vol citrate + 9 vol. blood).

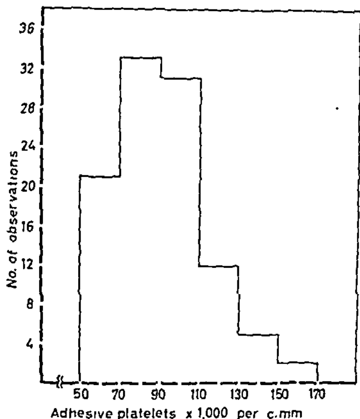


Fig 5. *Number of adhesive platelets in 104 samples of blood from 60 healthy persons*
The abscissa shows the number of adhesive platelets in citrated venous blood
(1 vol citrate + 9 vol blood).

The number of adhesive platelets averaged 90,000 per c.mm, with a range of 52,000 - 157,000 The distribution is shown in Fig. 5.

The average percentage of adhesive platelets was 42, with a range between 26 and 68 (Fig. 6).

Determinations were made 18 different times on one normal adult male during an 18-month period The platelet count varied between 157,000 and 214,000 with a mean of 184,000 The number of adhesive platelets showed a greater variation, ranging from 59,000 to 133,000 with a mean of 92,000 The percentage of adhesive platelets varied between 36 and 68 with a mean of 50.

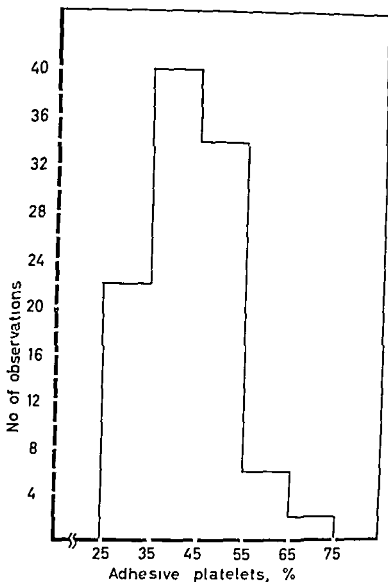
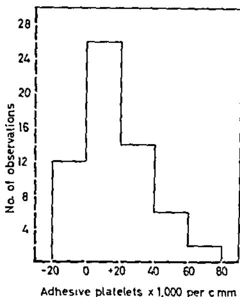


Fig 6. *Percentage of adhesive platelets in 104 samples of blood from 60 healthy persons.* The abscissa shows the percentage of adhesive platelets in citrated venous blood (1 vol citrate + 9 vol. blood).

Fig. 7. *Number of adhesive platelets in 60 samples of citrated plasma from 60 healthy persons.*



2. THE PLATELET ADHESIVENESS IN CITRATED PLASMA FROM HEALTHY PERSONS

The tests were performed on 60 citrated plasma samples from the same normal persons referred to under 1 Platelet-rich plasma was obtained from citrated whole blood by centrifugation at 190 G for 15 minutes The adhesiveness of the platelets was measured as soon as possible after the separation of the plasma.

The total platelet count in plasma showed a range of 238,000 - 569,000 with a mean of 380,000. The number of adhesive platelets showed a range of —17,000 to +65,000 with a mean of 15,000 as shown in Fig. 7. The negative values are due to counting errors when the true values lie close to zero

The percentage of adhesive platelets ranged from —4 to +15 with a mean of +4 (Fig. 8).

Discussion

The total platelet count observations confirm those of Sloan (1951). The number and percentage of adhesive platelets showed relatively great fluctuations both among different subjects and in one individual at dif-

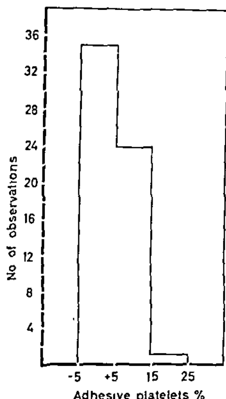


Fig. 8. Percentage of adhesive platelets in 60 samples of citrated plasma from 60 healthy persons

ferent times. The reason for the greater variation in the number of adhesive platelets is due, in part, to the fact that this number is a difference of two countings, each with its own counting error.

The investigation showed a normal range of 52,000 - 157,000 adhesive platelets per c.mm of citrated blood. In platelet-rich plasma, the platelet adhesiveness was nearly zero

The influence of red cells on the platelet adhesiveness

INTRODUCTION

The mechanism underlying the formation of the platelet plugs in haemostasis is still unknown. After microscopic section of a blood vessel, a platelet plug is observed within the first second in 45 per cent, and within the first two seconds in 73 per cent of the cases studied (Hugues 1959 b). Addition of high concentrations of citrate to the irrigating fluid inhibited the formation of platelet plugs in the divided small vessels in the mesenterium of rabbits, whereas less concentrated solutions did not. Injection of heparin (2,500 to 12,500 U/kg) did not inhibit the formation of the platelet plug (Hugues 1959 b).

In vitro, increasing concentrations of citrate decrease the platelet adhesiveness in whole blood. In concentrations greater than 30 mM, the platelet adhesiveness is zero (p. 33), whereas heparin does not prevent the adhesion of platelets to a glass surface (p. 35). These findings indicate that a possible relationship may exist between *in vivo* and *in vitro* platelet adherence.

Investigations of the mechanism of the platelet adhesiveness *in vitro* may therefore throw some light upon the *in vivo* mechanism of the adhesion of platelets to the vascular wall.

1. DEMONSTRATION OF A RED CELL SUBSTANCE (FACTOR R) WHICH AFFECTS THE PLATELET ADHESIVENESS

There is an almost linear correlation between the number of red cells in the plasma and the platelet adhesiveness (pp. 37-39). The presence of red cells therefore, is important for the immediate adhesion of platelets to glass. The alteration of the platelets in blood is possibly secondary to alterations of the red cells which have been in contact with a foreign surface. The correctness of this theory was tested by the following experiments.

Citrated blood from three normal persons was centrifuged at 190 G for 15 minutes. The platelet-rich plasma was pipetted off and set aside. The buffy coat was pipetted off and discarded. Equal volumes of the red cells remaining and saline were mixed well and then divided into two portions. One portion was passed through the glass bead column, then centrifuged at 160 G for 15 minutes. The supernatant was pipetted off and saved. The second portion was handled in the same way, but was not passed through the column. Two ml portions of the supernatant from the resulting suspensions were added to 2.5 ml of the platelet-rich plasma, and the platelet adhesiveness of the mixtures estimated. The results are given in Table 27.

Table 27. *The effect of the supernatant from a red cell-saline mixture on the platelet adhesiveness in plasma*

Super-natant	Sample no.	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
A	1	197	45	23
	2	228	88	39
	3	360	92	26
			Mean	29
B	1	197	12	6
	2	228	9	4
	3	360	1	0
			Mean	3

Two ml of the supernatant A or B was added to 2.5 ml of citrated platelet-rich plasma
Supernatant A was obtained from a red cell suspension which had been passed through a glass bead column, and B from a suspension which had not been in contact with glass beads

The platelet adhesiveness in the samples which had been mixed with supernatant from the suspension which had passed the column, amounted to 23-39 per cent, whereas the adhesiveness in the control experiments was only 0-6 per cent. These data indicate that, as a result of contact with the beads, the red cells release a water soluble substance which increases the adhesiveness of the platelets in citrated plasma.

2. THE LOCALISATION OF FACTOR R IN THE RED CELLS

An attempt to demonstrate the localisation of factor R in the red cells was made in the following experiments.

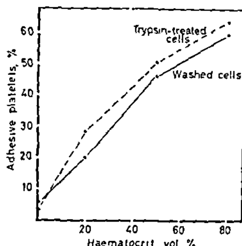


Fig. 9 *The effect of washed and of trypsin-treated red cells on platelet adhesiveness.*
The curve for trypsin-treated red cells is the mean of two experiments

A. The effect of washed red cells on platelet adhesiveness

Citrated blood from a normal person was centrifuged at 190 G for 15 minutes. The platelet-rich plasma was pipetted off and set aside The red cells were washed 4 times in large volumes of saline. The washed, packed red cells were added to the plasma in different proportions, to prepare samples with haematocrit values of 20, 50, and 80 volume per cent (= 2.2, 5.5 and 8.8 mill. red cells/c.mm) Plasma to which no red cells had been added served as a control. The platelet adhesiveness in each sample was estimated The results are shown in Fig 9. The platelet adhesiveness increased with increasing concentrations of washed red cells, as did the adhesiveness with increasing concentrations of unwashed red cells (Table 12, p 37). This demonstrates that factor R is not loosely adherent to the red cell surface.

B. The effect of trypsin-treated red cells on the platelet adhesiveness

Red blood cells from two normal persons were treated in the same manner as in the preceding experiment After the last washing with saline, one volume of packed red cells was suspended in 3 volumes of a solution containing trypsin (1 mg per ml) in M/15 phosphate buffer at pH 7.7. The mixture was incubated for half an hour at 37° C, and the cells again washed four times in large volumes of physiological saline The red cells

were mixed, in different proportions, with platelet-rich plasma from the same person, and the platelet adhesiveness was estimated.

The results are shown in Fig. 9. Washing with saline followed by treatment with trypsin did not remove or destroy factor R in the red cells. Thus, if it is of a protein nature, factor R is probably not located on the surface of the red cells.

C. The effect of lysed red cells on the platelet adhesiveness

Citrated blood was centrifuged at 190 G for 15 minutes, and the platelet-rich plasma was pipetted off and set aside. The red cells were centrifuged at 1,500 G for 10 minutes and the remaining plasma and buffy coat were pipetted off. The packed red cells were frozen at -25° C for 24 hours and subsequently thawed and frozen three times. The resultant haemolysate was diluted with three times its volume of physiological saline and centrifuged at 145,000 G for one hour. By this procedure, the supernatant was freed from red cell stroma (ghosts).

0.1 ml of the stroma-free haemolysate (final concentration of the haemolysate = 1:200) was added to 4.9 ml of the platelet-rich plasma, and the platelet adhesiveness was then estimated. The addition of haemolysate provoked a marked platelet clumping with a decrease in the platelet count from 390,000 to 203,000. The number of non-adhesive platelets was 50,000. This corresponds to 76 per cent adhesive platelets as calculated from 203,000 platelets, or 87 per cent as calculated from the original platelet count in the citrated plasma.

This result indicates that factor R is located in the inner structure of the red cells and is liberated at least in part by haemolysis.

D. The effect of red cell "ghosts" on the platelet adhesiveness

To find whether factor R is identical to haemoglobin or is released from red cells together with haemoglobin by haemolysis, the following experiments were made

Citrated whole blood was centrifuged at 190 G for 15 minutes. The platelet-rich plasma was pipetted off. The red cells were centrifuged for 10 minutes at 1,100 G. The supernatant plasma and buffy coat were removed. Packed red cell volumes of 1, 2, and 4 ml were placed in different siliconized tubes, frozen at -25° C for 24 hours, and thawed and frozen two more times. This haemolysate was mixed with three times its volume of saline and centrifuged at 1,100 G for 10 minutes. The resulting sediment

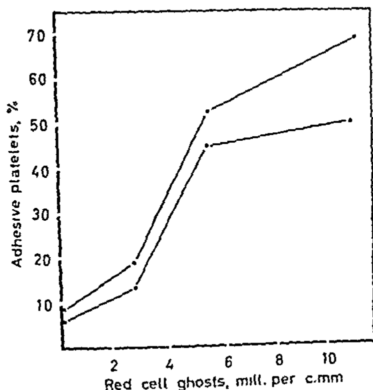


Fig. 10. *The effect of red cell ghosts on platelet adhesiveness*

of protein-like material was removed, and the supernatant from the different tubes centrifuged at 145,000 G (40,000 r p m.) for 30 minutes. The supernatants were decanted, and the sediments of red cell ghosts were resuspended in 10 ml portions of saline This washing was repeated six times. The final supernatants were translucent without any visible trace of haemoglobin. A minute greyish sediment of ghosts was visible at the bottom of the tubes

The sedimented ghosts in the different tubes were suspended in 4 ml of citrated platelet-rich plasma, and the platelet adhesiveness was tested immediately afterwards Due to difficulties in counting ghosts, the number in each plasma sample was calculated from the number of red cells present in the tubes before haemolysis Two such experiments were performed, and the results are shown in Fig. 10.

The platelet adhesiveness increased with increasing concentrations of red cell ghosts in a fashion similar to that noted with increasing concen-

trations of untreated, washed, and trypsin-treated red cells. These findings permit the following conclusions: Factor R is located on the stroma of red cells. Factor R is probably not identical with, or bound to, haemoglobin, since only three per cent of haemoglobin is left in the red cell ghosts after six washings (Anderson & Turner 1959). Factor R is only partly released into the solution by haemolysis of red cells

Summary

A substance is released from the red cells by contact with a foreign surface. This substance, which is called factor R, alters the properties of the platelets in citrated plasma. In presence of factor R, the non-adhesive platelets become adhesive. Factor R cannot be removed from the red cells by repeated washings in saline or by trypsin treatment. Red cell "ghosts" have the same effect on platelets as intact red cells Factor R probably is located on the stroma of the red cells and is only partly released into the solution by haemolysis. Factor R is probably not identical to haemoglobin.

3. THE EFFECT OF DIFFERENT AMOUNTS OF FACTOR R ON PLATELET ADHESIVENESS

A portion of the stock solution of factor R (p 70) was diluted 1:10 with saline. Different volumes of this solution, ranging from 0.013 ml to 0.416 ml, were pipetted into six different tubes, and saline was added to a final volume of 0.5 ml. Three ml of normal citrated, platelet-rich plasma was added to each tube, and the platelet adhesiveness was measured immediately afterwards. The results of two experiments are shown in Fig. 11. At low concentrations of factor R, there was an almost linear correlation between the platelet adhesiveness and the logarithm of the concentration At high concentrations of factor R, the curve flattened out, indicating that about 10 per cent of the platelets remain non-adhesive in the presence of factor R

4. PHYSICO-CHEMICAL PROPERTIES OF FACTOR R

The clarification of the chemical structure of a substance extracted from an organic compound is far beyond the scope of this work. Studies of some physico-chemical properties of factor R were, however, undertaken.

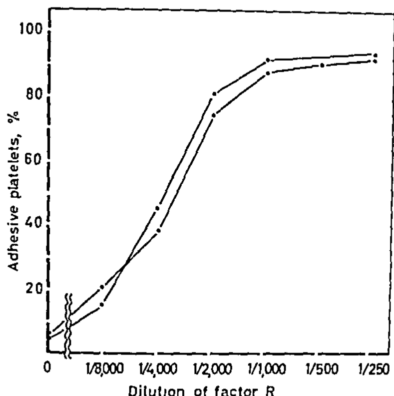


Fig 11. *The effect of increasing amounts of factor R on the adhesiveness of platelets in citrated plasma*

A. Heat stability of factor R

The heat stability of factor R was demonstrated in the following experiments Samples of six-times-washed red cell ghosts were prepared from 2 ml portions of packed red cells, and suspended in 2 ml saline. One portion was heated for 30 minutes at 100° C and then centrifuged at 145,000 G for 30 minutes. The supernatant was removed The other sample was not heated, but otherwise handled in the same way. The unheated and the heated ghosts were suspended in 4 ml of platelet-rich plasma, and the platelet adhesiveness measured

Table 28 shows that there was essentially no difference between the results obtained with the heated and unheated preparations, *i.e* factor R is heat stable.

Table 28. *The effect of heating on the ability of red cell ghosts to increase the platelet adhesiveness in citrated plasma*

	No. of ghosts in plasma, mill/c.mm	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
Control	0 0	369	3	1
Heated ghosts . ..	5.6	*	211	57
Unheated ghosts	5 6	*	181	49

If however, the supernatant from the heated ghost preparation was added to platelet-rich plasma, it could be demonstrated that the supernatant also greatly influences the platelet adhesiveness, whereas addition of the supernatant from the untreated ghosts was ineffective. This is shown in Table 29.

These experiments show that factor R is partly released into a saline solution by heating at 100° C for 30 minutes.

Table 29. *The effect on platelet adhesiveness of a saline extract prepared from red cell ghosts heated to 100° C*

Sample no	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
1	241	165	69
2	403	195	58
Control	280	10	4

[illegible]

B. The technique for preparation of factor R

The preparation of red cell ghosts is a time-consuming procedure In addition there is some loss of factor R due to a partial release of the factor into the solution during the haemolysing and washing procedures. Therefore, the following method for preparation of factor R was used.

One volume of packed red cells prepared from citrated blood is washed three times in two volumes of saline. After the third washing, the supernatant is pipetted off, and the red cells are suspended in two volumes of saline and transferred to an Erlenmeyer flask. The suspension is heated at 100° C for 15 minutes in a water bath, with occasional stirring. This boiled mixture is centrifuged for 20 minutes at 1,600 G. The supernatant (which still contains considerable amounts of haemoglobin) is transferred to another Erlenmeyer flask and again heated to 100° C for 15 minutes. After centrifugation at 1,600 G for 20 minutes the almost water-clear, faintly yellow supernatant is pipetted off and dispensed in 2 ml portions for storage at —25° C. The stock solution contains factor R "diluted 1:3," and is isotonic.

C. *The stability of factor R at -25° C and at room temperature*

Storage of a solution of factor R at —25° C for 8 months did not significantly influence its activity. When unsterile samples were stored at room temperature, the activity disappeared after 1-3 weeks. Therefore, solutions of factor R should be kept at —25° C.

D. *The influence of citrate and EDTA on factor R activity*

I previously suggested (pp. 63-64) that the platelet adhesiveness in whole blood *in vitro* depends upon the release of factor R from the red cells. As the platelet adhesiveness decreases with increasing concentrations of citrate and EDTA in whole blood (pp. 33-35), a demonstration that these substances similarly influenced "purified" factor R would support this theory further.

a. Citrate

To each of three portions of platelet-rich plasma from one donor, containing 18.2, 36.4 and 54.6 mM citrate per 1,000 ml respectively, factor R was added to a final dilution of 1:1,200. The platelet adhesiveness was estimated immediately afterwards. The results of two experiments are shown in Table 30. Increasing concentrations of citrate caused a progressive decrease of the effect of factor R on the platelet adhesiveness.

b. EDTA

When factor R was added to citrated, platelet-rich plasma containing 6 mM EDTA per 1,000 ml, no platelet adhesiveness was observed. In plasma containing the same amount of factor R but no EDTA, the number of

Table 30. *The effect of factor R on the platelet adhesiveness in the presence of increasing concentrations of citrate*

Sample no.	Plasma citrate conc., mM	Factor R in plasma, final dilution	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
1	18.2	1/1,200	405	276	68
	36.4	«	«	190	47
	54 6	«	«	58	14
2	18.2	«	377	228	61
	36.4	«	«	98	26
	54 6	«	«	6	2
Mean	18.2				65
	36.4				37
	54.6				8

adhesive platelets was high (see Table 31). The effect of citrate and EDTA in these experiments is similar to their effect on platelet adhesiveness in whole blood (cf. Tables 8 and 10). These findings suggest that factor R is responsible for the platelet adhesiveness in whole blood tested *in vitro.*

Table 31. *The effect of factor R on the platelet adhesiveness in the presence of EDTA*

Factor R in plasma, final dilution	Concentration of EDTA in citrated plasma, mM	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
1/225	0	320	221	69
1/225	6	320	—21	—7

E. Dialysis of factor R

Factor R was diluted 1:3 in saline Three ml of this solution were dialysed against 27 ml of physiological saline for 24 hours at 4° C. One tenth ml of the solution inside the membrane was added to 4 ml of freshly prepared platelet-rich plasma, and the platelet adhesiveness was estimated immediately afterwards. The fluid outside the membrane was tested in a similar experiment with plasma from the same donor In a third tube, 0.1 ml of a solution of factor R which had been diluted 1:30 and stored at 4° C for 24 hours, was tested in the same way. The results are shown

Table 32. *Dialysis of factor R.*

Contents of mixture, ml				Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
Platelet-rich plasma	Untreated factor R, diluted 1/30	Solution inside the dialyzing membrane	Solution outside the dialyzing membrane			
4	0.0	0.1	0.0	295	234	80
4	0.0	0.0	0.1	"	251	85
4	0.1	0.0	0.0	"	246	83

The tests were performed after dialysis for 24 hours (see text).

in Table 32. The platelet adhesiveness was identical in all three samples, *i.e.* the concentration of factor R in the fluid inside and outside the dialysis membrane was the same, and equal to that in the control solution

Two other experiments performed in the same way with a less concentrated solution of factor R gave identical results Thus factor R was found to be dialysable.

In a mixture containing plasma and saline which had been stored in the dialysis casing for 24 hours at 4° C the platelet adhesiveness was zero, which shows that the dialysis membrane did not contain substances which affected the platelet adhesiveness

F. Treatment of factor R with ion exchange resins

The factor R used in the following experiments was extracted from red cells with distilled water instead of saline in order to avoid possible interference of Na^+ and Cl^- ions with the resins Dowex 2 was used as anion exchange resin, and Dowex 50 as cation exchange resin (see Materials, p. 7 and Methods, p. 8). The ion exchange procedures were performed in Erlenmeyer flasks with an excess of the resins

Three ml portions of platelet-rich plasma from a normal donor were pipetted into three different tubes To the first tube was added 0.5 ml of the solution of factor R diluted 1 36 which had been treated with Dowex 2; to the second was added factor R solution diluted 1 36 and treated with Dowex 50; and to the third tube was added 0.5 ml of untreated factor R diluted 1·36. The platelet adhesiveness in the different mixtures was measured. The experiment was repeated with platelet-rich plasma from another donor The results are recorded in Table 33 Treatment of factor

Table 33. *The effect of anion and cation exchange resins on factor R activity*

Sample no.	Treatment of factor R	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
1	Dowex 2	354	64	18
	Dowex 50	"	327	93
	Untreated	"	336	95
2	Dowex 2	457	70	15
	Dowex 50	"	411	90
	Untreated	"	421	92

One half ml of factor R solution was added to 3 ml platelet-rich citrated plasma.

R with the cation exchange resin did not influence its effect on platelets, while factor R treated with the anion exchange resin had lost nearly all its ability to influence platelet adhesiveness.

Summary

Factor R is heat stable. By heating red cell ghosts in saline at 100° C, considerable amounts of factor R can be extracted. A simple technique for preparation of protein-free solution of factor R from washed red cells is given. Factor R solutions are stable for at least 8 months at -25° C.

The effect of factor R on platelet adhesiveness in citrated plasma is decreased with increasing concentrations of citrate and EDTA. Citrate and EDTA similarly influence platelet adhesiveness in citrated whole blood. It is suggested that factor R is responsible for the "immediate" platelet adhesiveness in whole blood tested *in vitro*.

Factor R is dialysable, and therefore of a relatively small molecular size After treatment with anion exchange resins factor R becomes ineffective.

5 THE EFFECT OF FACTOR R ON PLATELETS STORED AT 20° C IN CITRATED PLASMA

When platelet-rich plasma was stored at 20° C for 48 hours, the platelets did not regain their adhesiveness on subsequent storage at 4° C (pp 49-50). To find if storage of platelets influenced the effect of factor R on the platelet adhesiveness, the following experiments were made.

Portions of platelet-rich plasma from four persons were stored at 20° C. At intervals, factor R was added to a final concentration of 1:1,000, and

Table 34. *The effect of factor R on the adhesiveness of platelets which have been stored in citrated plasma at 20° C*

Sample no	Duration of storage at 20° C, hours	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
1	0	347	217	63
	2	•	256	74
	5½	•	218	63
	11½	•	149	43
	19½	•	36	10
2	0	314	120	38
	2	•	238	76
	6	•	227	72
	11	•	166	53
	18	•	121	38
3	0	469	236	50
	24	•	27	6
	48	•	0	0
	72	•	—3	—1
4	0	467	355	76
	24	•	—17	—4

• The final dilution of factor R in the platelet-rich plasma was 1/1,000

the platelet adhesiveness was estimated In two persons the estimations were performed at intervals of a few hours, and in the other two at intervals of 24 hours. The results are shown in Table 34

The effect of factor R was pronounced on platelets stored for about 6 hours. On further storage, a progressive decrease of the effect was observed. After 24 hours of storage, the platelets were not influenced by factor R. Storage of platelets in citrated plasma for at least 24 hours at room temperature therefore causes irreversible changes of their adhesiveness, which, as noted earlier (p 53), should be kept in mind in all platelet work.

6. THE INACTIVATION OF FACTOR R IN PLASMA

A. *The inactivation of factor R in platelet-rich plasma*

Different amounts of factor R were added to portions of platelet-rich plasma These portions were stored at room temperature, and the platelet adhesiveness was measured in each portion at intervals of five minutes. The results are shown in Fig. 12

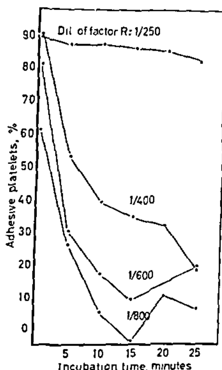


Fig. 12. *Inactivation of factor R in citrated platelet-rich plasma at room temperature.*

High concentrations of factor R exerted a constant effect on platelet adhesiveness during 25 minutes incubation, whereas lower concentrations *were rapidly inactivated in the same period.*

This inactivation of factor R might be caused by the platelets or the plasma. Therefore, in the next experiment, the platelets were removed before the incubation

B. The inactivation of factor R in platelet-poor plasma

Factor R was added to platelet-poor plasma (containing about 700 platelets per c.mm) to a final concentration of 1.300. Every 5-10 minutes one volume of this mixture was added to one volume of platelet-rich plasma from the same donor, and the platelet adhesiveness was measured immediately. The results of the experiments on plasma from 5 different normal persons are shown in Fig. 13.

From these data it was concluded that factor R was inactivated in platelet-poor as well as in platelet-rich plasma.

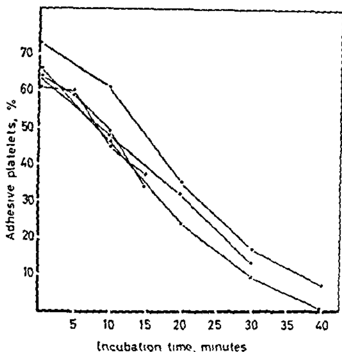


Fig. 13. *Inactivation of factor R in citrated platelet-poor plasma at room temperature*
Factor R was diluted 1/300 in platelet-poor plasma. Following incubation, an equal volume of platelet-rich plasma was added, and the platelet adhesiveness was tested in the usual manner.

7. THE EFFECT OF FACTOR R *IN VIVO*

Earlier investigations. Injection of haemolysed blood into dogs produced a haemorrhagic state and an increase of the whole blood clotting time (Nelson *et al.* 1950). In three of nine dogs injected with red cell haemolysate, Fogelman *et al.* (1952) found small hyaline emboli scattered throughout the lungs and haemorrhages from vessels of medium and small size. Coagulation studies showed variable results.

Krevans & Jackson (1955) reported a haemorrhagic diathesis in patients receiving massive whole blood transfusions. These patients always showed thrombocytopenia, the degree of which was dependent upon the amount of transfused blood and the rate of infusion.

Transfusion of incompatible blood produced haemoglobinaemia, thrombocytopenia, hypofibrinogenaemia and a bleeding tendency (Pifer *et al.* 1956, Krevans *et al.* 1957). Krevans *et al.* explained these alterations as the

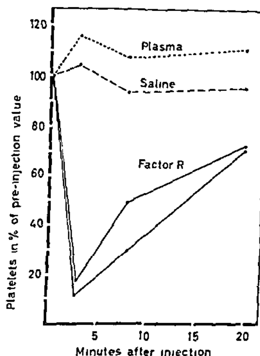


Fig. 14. *The effect of factor R in vivo* Rabbits were injected intravenously with saline, human plasma, and human factor R diluted 1/3 in saline. The platelets were counted in arterial blood.

result of intravascular coagulation caused by a thromboplastin-like activity of the haemolysed red cells. It seemed possible to explain the decrease in platelet number as due to the liberation of great amounts of factor R by the collection and transfusion of the blood, or by intravascular haemolysis

Personal investigation. To test whether factor R injected *in vivo* had any influence on the platelet number, two rabbits were injected with 10 ml of human factor R diluted 1:3 in saline As a control, each rabbit was injected on other days with 10 ml saline or with 10 ml of human plasma, diluted 1:3 in saline and deprived of its protein by heating twice to 100° C for 15 minutes. Each heating was followed by centrifugation at 1,000 G for 10 minutes. All solutions were injected intravenously as fast as possible (10 ml in about 20 seconds) At intervals, 1 ml blood was drawn from the central artery of the ear and the platelets counted by Nygaard's method. Platelet counts in capillary blood of the rabbits were unreliable due to a rapid decrease in the number of platelets in the blood from the wound. This decrease was probably due to removal of the platelets by coagulation of the blood, since in heparinized rabbits no such decrease was found.

The results of the investigation are shown in Fig 14. The platelet count decreased to 11 per cent and 17 per cent of the pre-injection values three

minutes after the injection. This decrease was followed by a rise to about 70 per cent during the next 20 minutes. The injection of the control solutions did not influence the platelet count. Thus the administration of factor R resulted in rapid, marked lowering of the platelet count.

8. IS FACTOR R PRESENT IN TISSUES OTHER THAN RED CELLS?

The presence of factor R in red cells raises the question whether similar substances are also present in other cells of the body

When extracts from other cells or tissues are prepared, any contamination with red cells must be avoided. Brain tissue may be prepared without significant contamination by blood, if handled as described by Hjort (1957, p. 17), for the preparation of thromboplastin. The walls of large vessels may also be practically freed from red cells before extraction.

A. Thromboplastin

Previous investigations. Bounameaux (1955 b), using Wright's method, found increased adhesiveness of rabbit platelets when rabbit thromboplastin was added to platelet-rich plasma, whereas the addition of rabbit thromboplastin to platelet-rich plasma from rats had little effect

Personal investigation An extract of human brain, obtained 36 hours after death, was prepared by the method of Hjort (1957) A portion of this crude suspension was centrifuged at 81,000 G for 30 minutes and the supernatant saved Another portion of the extract was heated to 100° C for 15 minutes, centrifuged at 81,000 G for 30 minutes and the supernatant saved Four ml of platelet-rich plasma was pipetted into each of four different tubes To the first tube 1 ml of the crude thromboplastin was added, to the second tube, 1 ml of the supernatant of the unheated thromboplastin; to the third, 1 ml of the supernatant of the heated thromboplastin, and to the fourth (the control), 1 ml physiological saline Table 35 shows that the platelet adhesiveness was nearly zero in all samples Thus brain tissue does not contain factor R

B. Crude cephalin

Crude cephalin was prepared by the method of Hjort *et al* (1955) Four ml of platelet-rich plasma was pipetted into each of three different tubes To the first tube 0 2 ml of the crude cephalin and 0 1 ml physio-

Table 35. *The effect of human brain thromboplastin on the adhesiveness of platelets in citrated plasma*

Sample no.	Test material	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
1	A	299	24	8
	B	"	22	7
	C	"	46	15
	D	"	55	18
2	A	414	—13	—3
	B	"	13	3
	C	"	4	1
	D	"	—7	—2

Four ml of platelet-rich plasma were mixed with 1 ml of the following materials:
A = crude thromboplastin
B = supernatant following high speed centrifugation of the crude thromboplastin
C = supernatant following high speed centrifugation of the heated crude thromboplastin.
D = saline.

logical saline were added; to the second tube, 0.3 ml of the crude cephalin; to the third (the control), 0.3 ml of physiological saline. The platelet adhesiveness was estimated in all samples Table 36 shows that cephalin had no effect on the platelet adhesiveness and thus contained no factor R.

Table 36 *The effect of different amounts of crude cephalin on the platelet adhesiveness in citrated plasma*

Contents of mixture, ml			Platelet count, × 1,000	Adhesive platelets, 1,000	Adhesive platelets, %
Platelet-rich plasma	Crude cephalin	Saline 0.9%			
4	0.2	0.1	392	10	3
4	0.3	0.0	392	0	0
4	0.0	0.3	370	—2	—1

C. *Saline extract of vascular wall*

The search for a factor R-like substance in the vascular wall is of particular interest, because its presence would explain why the platelets immediately adhere to the injured intima in thrombotic conditions and in normal haemostasis

Table 37. *The effect of saline extracts from bovine aorta on the platelet adhesiveness in citrated plasma*

Sample no.	Contents of mixture, ml				Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
	Platelet-rich plasma	Extract A	Extract B	Saline, 0.9%			
1	3	1	0	0	303	—14	—5
	3	0	1	0	•	12	4
	3	0	0	1	•	15	5
2	3	1	0	0	244	25	10
	3	0	1	0	•	30	12
	3	0	0	1	•	6	4
Mean	3	1	0	0			3
	3	0	1	0			8
	3	0	0	1			5

See text for description of extracts A and B.

The aorta of a cow was removed immediately after death, and thoroughly rinsed in tap water. As soon as possible it was brought to the laboratory, cut to pieces, and mashed in a meat-cutter. After addition of one volume of physiological saline, the suspension was ground for 15 minutes in a homogenizer. The suspension was centrifuged at 2,500 G for 15 minutes, and the supernatant was saved (extract A) The sediment was resuspended in twice its volume of distilled water, heated at 100° C for 15 minutes, and centrifuged at 2,500 G for 15 minutes. The resultant supernatant was mixed with an equal volume of 1 8 per cent saline solution (extract B). That addition of one ml of these extracts to samples of platelet-rich plasma did not increase the platelet adhesiveness, and thus, is presumed not to contain factor R, is shown in Table 37

As a control, factor R was prepared from the red cells of two cows. When the effect of these preparations on human platelets was compared with that of human factor R, it was found that each had an activity of about 70 per cent of the human factor R preparation The lack of effect in the above experiment, therefore, was not due to species differences.

Extracts from human vessel walls were not prepared

9. DEMONSTRATION OF AGGREGATION AND ADHESION OF PLATELETS TO GLASS IN THE PRESENCE OF FACTOR R

Previous investigations. Lundevall (1958, pp. 35-36) found that the addition of haemoglobin caused unspecific clumping of platelets in serum and in buffer solutions. This clumping could possibly have been due to factor R which was probably present in the haemoglobin solution.

Personal investigation

A. The effect of factor R on platelet clumping. When one drop of the stock solution of factor R was mixed with 1 ml platet-rich plasma in a tube at room temperature, the platelets started to aggregate 15-20 seconds afterwards After another few seconds the platelets formed large clumps which sedimented and adhered to the tube wall. If the concentration of factor R in the solution was below a certain limit, the clumps often disappeared after 10-20 minutes

Various degrees of platelet clumping caused by factor R are shown in Microphotograph No 6.

These experiments suggest that platelet adhesion and platelet clumping are related processes.

B The effect of factor R on platelet adhesiveness One drop of citrated platelet-rich plasma was deposited on a coverglass After tilting for 3-4 minutes the glass was rinsed with platelet-poor plasma Only a few platelets remained on the glass. If the platelet-rich and the platelet-poor plasma contained small amounts of factor R, numerous platelets adhered to the glass, as shown in Microphotographs Nos. 7 & 8.

Summary

The adhesiveness of platelets in freshly prepared citrated plasma increases in proportion to the logarithm of the concentration of factor R in the plasma, when this factor is present in low concentrations. However, if platelets are stored, in citrated plasma, at room temperature for 24 hours before addition of factor R, the increased adhesiveness resulting from factor R addition is lost. These findings indicate that the influence is exerted only on "viable" platelets

Factor R is inactivated *in vitro* by storage in platelet-poor citrated plasma.

Human factor R intravenously injected into rabbits causes an initial decrease of the platelet count to about 15 per cent of the pre-injection

value. This is followed by a rise, so that within 20-30 minutes the platelet count is 70 per cent of the pre-injection value. Thus it would appear that, *in vivo*, any factor R liberated is probably rapidly inactivated. This observation raises the question as to whether factor R is involved in the reactions following massive blood transfusions or sudden and massive intravascular haemolysis, since these include transient haemorrhages, thrombosis, and thrombocytopenia. However, a study of this problem is beyond the scope of the present work.

No factor R is found in extracts of human brain or bovine vessel wall. Therefore, factor R seems to be a fairly specific product of the red cells. However, white blood cells and platelets were not investigated for possible factor R content.

At low concentrations in citrated plasma, factor R increases the platelet adhesiveness. At higher concentrations factor R provokes marked platelet clumping. These findings indicate that the processes of platelet adherence to foreign surfaces and platelet aggregation are closely related.

Platelet adhesiveness in bleeding disorders

INTRODUCTION

In the literature, only a few instances are reported of the estimation of platelet adhesiveness in cases of haemorrhagic disorders. The results have been conflicting, possibly because different methods were used for the estimation.

The investigations reported here were performed on patients with different haematologic disorders. Most of the tests were performed more or less as a routine during the hospitalisation of the patients. A systematic examination of large groups of patients with various disorders has not been performed so far. Therefore, the number of patients in the individual groups varies considerably, and perhaps groups including only one or two patients should not have been reported before a larger number had been examined. However, the investigations in some of these cases point to problems of interest in relation to the mechanism of haemostasis. Further studies are in progress and will be published later.

1. THROMBASTHENIA

Earlier observations Thrombasthenia (Thrombasthenia haemorrhagica hereditaria, Glanzmann-Naegeli) is in this work defined according to Braunsteiner (1955), as a haemorrhagic disorder due to a qualitative platelet defect. The clinical picture is dominated by haemorrhages from mucous membranes, joint bleedings are seldom observed The bleeding time is prolonged, the clot retraction is reduced or absent, and the platelet count is normal The capillary fragility test is positive in some cases The platelets do not spread out or adhere on contact with foreign surfaces, nor do they form pseudopodia Thromboplastin generation, prothrombin consumption, and all known coagulation factors are within normal limits (Braunsteiner & Pakesh 1956). The quantitative estimation of platelet adhesiveness in

thrombasthenia has been reported in only one patient and in this patient it was zero (Marx & Derlath 1957).

Personal observations. Three patients who fulfilled the diagnostic criteria of Braunsteiner were observed. All patients had a normal platelet count, a prolonged bleeding time, a normal prothrombin consumption, a normal thromboplastin generation, and normal coagulation factors as judged from the Quick test, the P & P estimation, and the cephalin time. The clot retraction was absent in one case (I. S J.) and greatly reduced in the other two Fatal bleeding had occurred in the families of two of the patients (T. U. & S B.).

The platelet adhesiveness in citrated whole blood was measured twice in one case, and once in each of the two remaining cases (Table 38). The platelet adhesiveness was markedly reduced in all three patients The lowest number of adhesive platelets was found in the patient who had no clot retraction (I. S. J.). This patient also had the most severe clinical picture, with multiple bleeding episodes requiring repeated blood transfusions.

Table 38 *The platelet adhesiveness in citrated whole blood from three patients with thrombasthenia*

Patient	Sex	Age, years	Bleeding time (Ivy), min.	Haematocrit, vol. %	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
S B	male	12	>30	38	235	9	4
•	•	•	>30	38	213	6	3
T. U.	female	3½	12(Duke)	30	345	26	8
I S. J	•	16	>30	32	196	3	1

The patient I. S. J. was investigated at an early stage of this work by a modification of the method of Moolten & Vroman (1949 a), using a "glass filter" consisting of 10 g of glass beads instead of glass wool. One ml of citrated blood was placed in the "glass filter", which had previously been moistened with saline. Thirty seconds later, the blood was washed out of the filter with 5 ml of saline. The dilution of the blood was estimated from the decrease in the red cell count before and after the passage through the column. The number of platelets found after the passage through the column was corrected according to the dilution, and the number of platelets adhering to the beads was calculated (Table 39, A). As a control, platelet adhesiveness was tested in blood samples from five normal persons, using the same method (Table 39, B) In addition, the platelet adhesiveness

Table 39 *The influence of saline on the measurement of platelet adhesiveness in thrombastheni*

	Unfiltered sample		Filtered sample		Non-adhesive platelets, corrected for dilution, × 1,000	Adhesive platelets, × 1,000	Adhesiv platelets %
	Red cells, mill	Platelets, × 1,000	Red cells, mill.	Platelets, × 1,000			
A	2.80	196	1 43	49	96	100	51
	2 80	196	1.35	45	93	103	53
B	3.94	187	1 13	21	73	114	61
	3 96	168	1.03	22	85	83	50
	3 59	259	1 11	26	84	175	68
	3 79	280	1.03	29	107	173	62
	3 85	300	0 95	41	164	136	45
C	2 80	196		181		5	3
	2 80	196		196		0	0
D		280		111		169	61
		300		137		163	54
		262		122		140	53
		188		86		102	54
		199		116		83	42

citrated whole blood *from*
A. Vroman,
B.
C.
D. the five normals without the use of saline

was measured in patient I S J and in five normal persons by running 5 ml of the citrated whole blood through the glass bead columns without using saline (*Table 39, C and D*)

The results were surprising When tested with saline, the platelet adhesiveness in the thrombasthenic patient was within the normal range If saline was not used, however, the adhesiveness of the thrombasthenic platelets was zero and differed markedly from that of the normal platelets. This shows that the method of Moolten & Vroman, as modified, does not give reliable results for thrombasthenic platelets. The duration of the contact time between blood and glass beads was nearly the same in both experiments, therefore the difference in adhesiveness was probably due to the influence of saline.

The adhesiveness of thrombasthenic platelets suspended in saline

Platelets from 5 ml of platelet-rich plasma from the patient S B. were washed once and then resuspended in 5 ml of saline. The adhesiveness of the platelets was measured by the author's method. The result is presented in Table 40. Also shown is the platelet adhesiveness in citrated plasma from the same patient.

Table 40. *The adhesiveness of platelets from a patient with thrombasthenia (S B.)*

	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
A	469	—1	0
B	297	72	24
C		(244)	(52)

A. in platelet-rich plasma,
B in saline,
C. in saline as in B, but the platelets lost during washing and resuspension are here included in the figure for adhesive platelets

Platelets in saline were prepared from 5 ml citrated platelet-rich plasma After centrifugation for 20 minutes at 1,800 G, the platelets were resuspended in 5 ml saline, centrifuged again, and resuspended in 5 ml saline.

This experiment shows that, when suspended in saline, the adhesiveness of thrombasthenic platelets is artificially increased The platelet count decreased during washing and resuspension of the platelets. Since the discarded supernatants were found to be free of platelets, this diminution in number probably resulted from the adherence of platelets to the glass wall of the tube. If this loss is included in the figure for adhesive platelets (c), a normal result is obtained. Therefore, saline should not be used in estimations of platelet adhesiveness

The effect of storage at 4° C on thrombasthenic platelets

Platelet-rich plasma from patient S. B. was stored at 4° C for three days, and the platelet adhesiveness measured daily Table 41 shows that thrombasthenic platelets, suspended in plasma, do not become adhesive on storage at 4° C, in contrast to normal platelets (pp. 48-49)

The effect of factor R on thrombasthenic platelets

The exact nature of the platelet defect in thrombasthenia is not known. Therefore, it was of interest to test the influence of factor R on the adhesiveness of thrombasthenic platelets Factor R, prepared from the red cells

Table 41. *The effect of storage at 4° C on the adhesiveness of thrombasthenic platelets in citrated platelet-rich plasma*

Storage at 4° C, hours	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
0	469	—1	0
24	473	0	0
48	446	20	4
72	489	10	2

of a normal subject was added to the platelet-rich plasma from patient S. B. to a final concentration of 1/75. The platelet adhesiveness was tested immediately afterwards. Normal platelet-rich plasma, treated in the same way, served as control. In a third experiment, factor R, prepared from the red cells of the patient, was added to platelet-rich normal plasma to a final concentration of 1/75.

Table 42. *The effect of factor R on normal and thrombasthenic platelets*

	Platelet-rich plasma	Factor R	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
A	Patient	Normal	468	10	2
B	Normal	Normal	387	291	75
C	Normal	Patient	337	281	84

In all experiments 0 2 ml of a solution of factor R, diluted 1/3 in saline, was added to 4 8 ml of citrated platelet-rich plasma.

Table 42 shows that factor R prepared from normal red cells does not increase the adhesiveness of thrombasthenic platelets These platelets there*fore differ from normal platelets in this respect However*, a normal amount of factor R was found in the red cells of the thrombasthenic patient. From the data presented, it seems likely that the defect in thrombasthenia is releated to the resistance of thrombasthenic platelets to factor R, and may not be attributed to a lack of factor R.

2. HAEMOPHILIA A WITH PROLONGED BLEEDING TIME (V. WILLEBRAND-JÜRGENS' DISEASE)

During the last few years a "new" haemorrhagic disease, characterized by a prolonged bleeding time and a deficiency of the antihaemophilic globulin (AHG, factor VIII), has been described. Since AHG deficiency

has been recently observed in v. Willebrand's disease (v. Willebrand-Jurgens' thrombopathia) by Jürgens *et al.* (1957) and by Nilsson *et al.* (1957 a), several authors have suggested that v. Willebrand's disease and the "new" disease are identical (Bernard *et al.* 1957, Nilsson *et al.* 1957 b, Deutsch 1957, Biggs & MacFarlane 1958).

The haemostatic defect in the "new" disease is still obscure. It may be a vascular abnormality, plasmatic defect, qualitative platelet defect, or some combination of these. It is difficult to show which theory is correct, since reliable tests for vascular defects are not available, and tests for platelet defects depend heavily on such techniques The following example is illustrative of some of the difficulties encountered: Nilsson *et al.* (1957 b) found a normal thromboplastin generation test with platelets from patients in the Aaland islands, whereas Jurgens & Schultz (1958) found pathological values in the same patients, using a slightly modified method

Evidence of a platelet defect has been reported by several authors. Jürgens & Forsius (1951) found that platelets from such patients had a reduced ability to aggregate. Schultz *et al* (1958) found that the platelets were morphologically abnormal when examined by electron-microscopy. Increased osmotic resistance of the platelets from patients with v. Willebrand's disease was found by Ulutin & Karaca (1958). Nilsson *et. al.* (1957 b), however, believe that the disease is caused by a plasmatic defect, because they were able to correct the abnormal bleeding time by injections of Cohn's fraction I-0.

The problem whether a platelet defect is involved in the pathogenesis of the disease is therefore still unsolved, and a quantitative estimation of platelet adhesiveness might be of interest

Previous investigations. Marx & Derlath (1957) found a normal platelet adhesiveness in one patient with v Willebrand's disease

Personal investigation. Fifteen patients with AHG deficiency and a prolonged bleeding time were examined (Table 43) In four of these patients (1-4), the reduction of AHG was verified by the demonstration of a prolonged cephalin time (Waaler 1957), which was corrected by the addition of adsorbed plasma In the remaining 11 patients, the AHG content of the plasma was estimated by the method of Waaler (1959 b).

In patients whose plasma contained 10 per cent or less of the normal amount of AHG, a markedly prolonged bleeding time was found. Five patients (10, 11, 12, 14 and 15) had more than 10 per cent of the normal AHG content. Two of these patients (14 and 15) had a bleeding time longer than 30 minutes in spite of their comparatively high AHG content These patients had a reduced platelet count.

Table 43 *Platelet count and adhesiveness in 15 patients with prolonged bleeding time and AHG deficiency (von Willebrand's disease)*

Patient no	Age, years	Sex	Bleeding time (Ivy), min.	AHG, %	Haematocrit, vol. %	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
1	15	F	>20	—	34	240	45	19
2	2	M	>15	—	30	472	118	25
3	6	M	>20	—	39	246	102	42
4	3	M	>20	—	34	317	96	34
5	5	M	>30	3	26	395	77	20
6	9	F	>30	10	37	271	78	29
7	37	F	>30	10	41	175	59	34
8	25	F	>30	10	41	212	44	21
9	23	M	>30	4	45	265	114	43
10	22	M	13	45	46	216	69	32
11	14	F	12.5	29	38	237	81	34
12	22	M	12	49	45	209	57	27
13	15	F	>40	4	32	188	30	16
14	13	F	>30	41	40	109	48	44
15	33	M	>30	12	46	114	45	37

The percentage of adhesive platelets was normal (between 26-68 per cent) in 10 patients. In five patients (1, 2, 5, 8 and 13) values were below the normal range; only one (8), however, had a normal haematocrit value The reduced platelet adhesiveness in patients 1, 2, 5 and 13 (?), therefore, may be explained by the low haematocrit values Thus altered adhesiveness does not seem to be a factor in the pathogenesis of this disorder.

3. THROMBOCYTOPENIA

Previous investigations. In two patients with idiopathic thrombocytopenic purpura (ITP), Moolten & Vroman (1949 c) found 8 per cent adhesive platelets in one case and no adhesive platelets in the other Weiner *et al.* (1948) found a slight decrease in the number of adhesive platelets in one case (Wright's method) Braunsteiner (1951) showed that platelets from thrombocytopenic patients were unable to form pseudopodia and to spread out on foreign surfaces Plasma from a patient with platelet antibodies also inhibited these functions in normal platelets (Braunsteiner & Pakesch 1954). Bounameaux (1955 c) found a marked decrease of platelet adhesiveness in four patients with ITP, and demonstrated that normal platelets lost their adhesive property when suspended in the plasma from a patient with "allergic thrombocytopenia"

Personal investigation. Nine patients with ITP were investigated. All patients had a prolonged bleeding time. Platelet count and platelet adhesiveness were estimated in these patients and the results are shown in Table 44.

Table 44. *Platelet count and adhesiveness in nine patients with idiopathic thrombocytopenic purpura (ITP)*

Patient no	Bleeding time (Ivy), min.	Haematocrit, vol. %	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
1	>20	25	8.1	0.5	6
2	>60	38	10.5	4.2	40
3	>30	38	9.2	4.1	45
4	>30	42	89.5	36.2	41
5	>30	41	60.3	28.8	48
6	>30	40	17.0	8.5	50
7	>30	43	58.3	34.5	59
8	>30	36	80.0	23.2	29
9	>30	45	31.9	21.5	67

Seven of the patients had a normal percentage of adhesive platelets. Platelet adhesiveness was above normal in one patient (9) and markedly below in another patient (1). These findings do not agree with those of the earlier authors, but are in accordance with the finding of Lewalle *et al.* (1959), that "the resistance of the haemostatic plug is normal in thrombopenic patients". There may be several explanations for the discrepancy. One is that "ITP" may include diseases of different etiology. Another explanation is that different techniques have been used for estimation of platelet adhesiveness. Thirdly, some variation in platelet adhesiveness may occur during the course of the disease.

4 CONGENITAL AND ACQUIRED DEFECTS OF COAGULATION FACTORS

Patients with coagulation defects generally suffer from a haemorrhagic diathesis characterized by late or "after"-bleedings. In these patients, however, the bleeding time is not prolonged (Borchgrevink & Waaler 1958). A normal platelet adhesiveness therefore would be expected in these patients.

Previous investigations. Wright (1946) found decreased platelet adhesiveness in four cases of haemophilia. Spooner & Mayer (1944) found nor-

mal values in one haemophiliac, as did Weiner *et al.* (1948) in two patients. In these two cases, however, platelet adhesiveness increased after correction of the coagulation time by "antihaemophilic fraction I". Marx & Derlath (1957) found normal platelet adhesiveness in one patient with haemophilia A and in one with haemophilia B (Christmas disease).

Spooner & Mayer (1944) found decreased platelet adhesiveness in 33 patients treated with dicoumarol. This was confirmed by Wright (1945) on rabbits.

Personal investigation. One patient with haemophilia A, one with haemophilia B (Christmas disease), one with congenital prothrombin deficiency (Borchgrevink *et al.* 1959), one with congenital proaccelerin deficiency (Owren 1947), two with proconvertin deficiency (Aas 1952), and two with phenylindanedione-induced hypocoagulability of the blood were investigated. Of the drug-induced hypocoagulabilities one patient had a P & P value of 25 per cent, the second had an extremely low value (less than 2 per cent) after attempted suicide by ingestion of phenylindanedione. All patients had a normal bleeding time (less than 11 minutes). Observations on platelet adhesiveness in these patients are given in Table 45.

Platelet adhesiveness was normal in all patients. These findings are in contrast to those of Spooner & Mayer (1944) and of Wright (1945),

Table 45 *The platelet count and adhesiveness in congenital clotting disorders and in patients on anticoagulant treatment with phenylindanedione (P I D)*

Patient	*Age, years*	*Sex*	Haematocrit, vol. %	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %	Diagnosis
E M.	61	M	45	252	136	54	Haemophilia A (12%)
I.O M	16	M	33	257	94	37	Haemophilia B (<1%)
T T.	38	F	29	212	87	41	Prothrombindef (9%)
M. B.	45	F	46	208	97	47	Proacceleríndef (<1%)
S. K.	10	F	36	185	60	31	Proconvertindef (<3%)
A. M	46	M	39	262	115	44	Proconvertindef (<3%)
N. R.	25	M	44	351	97	28	P.I D (PP = 25%)
M. W.	50	M	44	246	106	43	P I.D (PP < 2%)

possibly because a different technique was used. During the prolonged contact between blood and glass in Wright's rotating chamber, some coagulation factors probably are activated and then may influence the adhesiveness of normal platelets. At low concentration of coagulation factors, less activation occurs and therefore platelet adhesiveness is increased less. The discrepancy indicates that the platelet adhesiveness as measured by Wright's method is not identical to the "immediate" adhesiveness which is measured by the present method. A normal platelet adhesiveness in these patients is in better agreement with the normal bleeding time than the decreased adhesiveness observed by Wright's method.

5. PERNICIOUS ANAEMIA

In pernicious anaemia, prolonged bleeding time, poor clot retraction and purpura, as well as retinal haemorrhages may be found (Wintrobe 1951, p. 447). Alteration of the platelet morphology as well as a decreased platelet count have been observed (Arneth 1937). Reviewing the literature, Paddock & Smith (1939) found among 93 cases 73 (78 per cent) with a platelet count below 100,000 per c.mm. Among their own 22 untreated patients with a red cell count below 2 mill. per c.mm, the platelet count was below 100,000 in 20 (90 per cent). During specific treatment, the platelet count increased to normal.

Personal investigation

A woman with pernicious anaemia, 62 years old, was investigated prior to the treatment with vitamin B_{12}, and 3 and 4 weeks after the start of treatment. The result of the investigation is shown in Table 46.

The patient had a red cell count of 1.7 mill. per c.mm, a low platelet count, and a prolonged bleeding time prior to therapy. Following specific treatment, both platelet count and the bleeding time became normal.

It is of more interest, however, that the investigation possibly unveils the mechanism of the prolonged bleeding time in this case. Prior to the treatment, the bleeding time was 32 minutes and the platelet count was 106,000. This slight thrombocytopenia should not cause a prolonged bleeding time, since O'Brien (1951) found a normal bleeding time in patients with platelet counts above 100,000 per c.mm.

A qualitative platelet defect therefore might have been present. This presumption was supported by the fact that the platelet count was normal three weeks after the start of the treatment, but the bleeding time was

Table 46. *The platelet count and adhesiveness, haematocrit, and bleeding time in a patient with pernicious anaemia before and during treatment with vitamin B 12*

	Bleeding time (Ivy), mins.	Haemato-crit, vol. %	Platelet count, × 1,000	Adhesive platelets, × 1,000	Adhesive platelets, %
Before treatment	*32*	*20* (45)	*106* (112)	*11* (57)	*10* (51)
After 3 weeks of treatment	20	30 (45)	171 (123)	33 (71)	19 (58)
After 4 weeks of treatment	8	38 (45)	268 (217)	105 (115)	39 (53)

The figures in brackets give the results obtained in blood samples artificially corrected to a haematocrit of 45% by removal of calculated amounts of platelet-rich plasma.

still prolonged. The bleeding time did not become normal until the number of adhesive platelets was within the normal range. This finding suggests that the prolonged bleeding time in this case was due to a reversible hypo-adhesiveness of the platelets. However, no absolute qualitative platelet defect could be demonstrated. Further study showed that 50 per cent of the platelets became adhesive when the haematocrit was regulated to 45 per cent *in vitro* (p. 38), both before and during treatment (values in brackets, Table 46). The decreased adhesiveness, possibly reflected only the patient's anaemia, indicating that the red cells influence the haemostasis, as postulated by Duke (1910).

6. THE RELATIONSHIP BETWEEN NUMBER OF ADHESIVE PLATELETS AND THE BLEEDING TIME IN PATIENTS WITH DECREASED OR VARIABLE PLATELET ADHESIVENESS

The complete mechanism of haemostasis is still unknown. Several factors are involved which include coagulation, vascular functions, and platelet functions. To study the significance of platelet adhesiveness in haemostasis, one should select patients without clotting or vascular defects. Even in these cases it is difficult to demonstrate a direct relationship between bleeding tendency and platelet adhesiveness for the following reasons:

1. The bleeding tendency is evaluated from the bleeding time, which is not an accurate test. Therefore, only a rough correlation between the number of adhesive platelets and the bleeding time may be expected.

2. Usually, the percentage of adhesive platelets is rather constant in one individual. Therefore, during fluctuations of the platelet count, the bleeding time may be correlated with both the number of adhesive platelets and the platelet count. The influence of platelet adhesiveness therefore can be demonstrated best in patients who have a constant platelet count close to the critical level of about 100,000 per c.mm, and yet variation in the number of adhesive platelets. Such patients are rare, and only one patient fulfilled these criteria.

Case report: E. H., 36 years, female, married For the last seven years, she had suffered from cyclic trombocytopenia with intermittent purpura and severe menstrual bleedings. The clotting factors were normal Two years after the onset of the disease, splenectomy was performed without significant effect on the disease. Steroid treatment, likewise, had no effect.

Each thrombocytopenic period lasted for about one week, followed by a thrombocythaemic phase of 2-3 weeks. No platelet antibodies were demonstrable in her blood (method of Dausset, 1954) When 300 ml of her plasma was collected during a thrombocytopenic episode and reinjected during a thrombocythaemic phase, no thrombocytopenia occurred

Platelet count, platelet adhesiveness, and bleeding time determinations were performed on samples taken during three episodes of thrombocytopenia and two periods of thrombocythaemia During the first thrombocytopenic period, platelet adhesiveness was markedly decreased In the following two periods of thrombocytopenia, however, the platelets were normally adhesive. Platelet adhesiveness was normal in one of the thrombocythaemic periods, and decreased in the other

There was a fairly good inverse correlation between platelet adhesiveness and the bleeding time during the different phases No such correlation was observed between the bleeding time and the platelet count This is illustrated in Table 47, which shows the bleeding time and number of adhesive platelets on selected days from the three different thrombocytopenic episodes, where almost identical platelet counts just above the critical level of 100,000 per c.mm were found

Studies were conducted on 13 occasions, including the seven in Table 47. A bleeding time of 12 minutes was found three times The corresponding adhesive platelet determinations ranged from 18,000 to 37,000 per c mm On six occasions a bleeding time of 5-6 minutes was recorded In five of these instances, the number of adhesive platelets ranged between

Table 47. *Platelet count, number of adhesive platelets, and bleeding time in one patient with cyclic thrombocytopenia*

	Date of investigation						
	3-10-56	10-12-56	25-3-57	12-11-56	16-11-56	1-3-57	4-3-57
	Thrombocytopenic states			Thrombocythaemic states			
Platelet count, × 1,000	103	126	129	733	837	1,047	810
Adhesive platelets, × 1,000	13	57	62	326	415	175	143
Bleeding time (Ivy), min	23	5.5	6	2	1.5	5.5	5.5

57,000 and 175,000 per c mm (*i. e.* within the normal range). In the sixth instance the number of adhesive platelets was 32,000 per c.mm

A prolonged bleeding time, which coincided with decreased numbers of adhesive platelets, was observed four times. The lowest value of adhesive platelets recorded was 13,000 per c.mm. On this occasion the bleeding time was 23 minutes, in spite of a platelet count above 100,000 per c.mm In thrombocythaemic periods, the shortest bleeding time recorded corresponded to the highest number of adhesive platelets and not to the total number of platelets The critical level of adhesive platelets necessary to keep the bleeding time below 12 minutes seemed to be between 20,000 and 40,000 per c.mm

Table 48 *Platelet count, number of adhesive platelets, and bleeding time in four patients with decreased platelet adhesiveness and prolonged bleeding time*

Patient	Date of investigation	Bleeding time (Ivy), mins	Platelet count, × 1,000	Adhesive platelets, × 1,000	Diagnosis
R. O	22-11-56	15	168	43	Metrorrhagia
R O	6-12-56	6	194	64	
R. O.	19-12-56	12	157	42	
A. R.		30	135	33	Metrorrhagia
O. N.		17	260	39	Haematuria
H. M		23	483	35	Thrombocythaemia

In addition to these observations, single observations on three patients, and three observations on one additional patient, gave further support to the establishment of a correlation between the bleeding time and the number of adhesive platelets In all these cases a prolonged bleeding time was found when the platelet count was above 100,000 per c.mm.

The platelet counts, numbers of adhesive platelets, bleeding times, and diagnoses are given in Table 48.

In all cases there was a fairly good inverse correlation between the number of adhesive platelets and the bleeding time. Of particular interest is the finding of a normal bleeding time in the patient R. K. on an occasion when the number of adhesive platelets was within the normal range.

Summary

The platelets of three patients with thrombasthenia were non-adhesive in citrated whole blood In saline, however, the thrombasthenic platelets were markedly adhesive. The use of saline in methods for estimation of platelet adhesiveness should therefore be avoided Thrombasthenic platelets suspended in plasma did not show increased adhesiveness when stored at 4° C as normal platelets do. The adhesiveness of thrombasthenic platelets in citrated plasma was not influenced by factor R Red cells from thrombasthenic patients contained a normal amount of factor R. The basic defect in thrombasthenia is therefore a platelet defect. The adhesiveness of thrombasthenic platelets in saline is possibly an artifact and does not prove the presence of an inhibiting factor in plasma as postulated by Braunsteiner & Pakesh (1956).

Of 15 patients with haemophilia A and a prolonged bleeding time (v. Willebrand-Jurgens' disease), 14 (13?) showed a normal platelet adhesiveness. Only one patient showed a significant reduction of the platelet adhesiveness. These observations indicate that the prolonged bleeding time in this disease is not due to decreased platelet adhesiveness

In nine patients with ITP, the percentage of adhesive platelets was found to be normal in eight and markedly reduced in one.

The platelet adhesiveness was found to be normal in patients with congenital and acquired defects of coagulation factors (haemophilia A and B, prothrombin-, proaccelerin-, and proconvertin-deficiencies, and phenylindanedione-induced hypocoagulability) These findings are in better agreement with the normal bleeding time in these patients than with the decreased adhesiveness reported by authors using the method of Wright in haemophilia and in drug-induced hypocoagulability This discrepancy

indicates that the platelet adhesiveness which is measured by the present method is not identical with the adhesiveness measured by Wright's method.

In one case of pernicious anaemia, an inverse correlation between the bleeding time and the number of adhesive platelets was found, but there was no correlation between the bleeding time and the total platelet count. Tested in whole blood, the platelet adhesiveness was greatly reduced before and during the first weeks of treatment. If *in vitro* correction of the haematocrit was made before measurement of the adhesiveness, however, the platelet adhesiveness was found to be within the normal range. This finding suggests that the formation of a platelet plug *in vivo* is influenced by the red cells in the blood stream. Further studies on this important problem are necessary, however.

In one case of cyclic thrombocytopenia with varying platelet adhesiveness and in four patients with decreased platelet adhesiveness, a fairly close inverse correlation between the number of adhesive platelets and the bleeding time was demonstrated, whereas no correlation between the bleeding time and the total number of platelets was found. The findings in these patients further support the suggestion that there is a correlation between the number of adhesive platelets and haemostasis.

Discussion

1. THE METHOD

The direct microscopic observation of *human* platelets *in vivo* is technically very difficult, and investigations on the adhesiveness of human platelets therefore rely upon observations *in vitro*. Among the few earlier methods, only those of Wright (1941) and of Moolten & Vroman (1949) have been widely applied. Technical errors, however, are inherent in these methods.

In Wright's method, platelets adhere to the wall of a rotating glass bulb, and the contact time between platelets and the foreign surface is very long (20-80 minutes). Zucker (1949) studied normal haemostasis in man by examination of skin biopsies and found that the platelet plug was formed within 10-30 seconds after injury By direct observation on rabbit vessels, Roskam *et al.* (1955) found that the platelets started to adhere to the injured vessel wall without delay. Hugues (1959 b) found, in rabbits, that the platelet plug started to form within 1-3 seconds Estimation of the platelet adhesiveness by prolonged contact time, therefore, may reflect properties of the platelets which are not responsible for the process of immediate platelet adherence *in vivo*

With Wright's method, a decreased platelet adhesiveness was found in dicoumarol-treated patients (Spooner & Mayer 1944), in dicoumarol-treated rabbits (Wright 1945), and in patients with haemophilia (Wright 1946) This indicates that coagulation factors influence the adhesiveness when the contact time is prolonged In the present method, the contact time is standardized at 30 seconds By this method, platelet adhesiveness was found to be normal in patients with haemophilia and various other coagulation disorders (pp 91-93). These findings indicate a qualitative difference between the platelet adhesiveness measured by short and by prolonged contact times

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Moolten & Vroman eliminated the error of prolonged contact time by increasing the glass surface area considerably, but they introduced two new sources of error in the method. First, they ignored standardization of the glass area, since they used a hand-made glass braid as a foreign surface. As shown in the present work (p. 32), there is, within certain limits, a close correlation between the number of adhesive platelets and the glass surface area. Secondly, Moolten & Vroman's method included dilution of the blood with large amounts of saline and citrate solutions. Marx & Derlath (1957) have shown that platelets in saline have "their own adhesiveness". Braunsteiner (1955) found that platelets from patients with thrombasthenia, while not adhesive in whole blood, became adhesive in saline. In the present work it is shown (pp. 53-55) that the adhesiveness of platelets in saline is not eliminated by EDTA, in contrast to the adhesiveness of platelets in whole blood. Further, it is demonstrated (pp. 85-86) that thrombasthenic platelets have a normal adhesiveness when tested by a slightly modified method of Moolten & Vroman, but that the adhesiveness is zero when tested in the absence of saline. These observations show that the adhesiveness measured by Moolten & Vroman's method is not identical with the platelet adhesiveness in citrated whole blood.

The literature concerning the platelet adhesiveness in different disorders contains conflicting results depending upon the method which has been used. However, conflicting results have also been reported by authors using identical methods. These points are illustrated in Table 49. These

Table 49 *Previous reports on platelet adhesiveness*

Groups of patients	Author	Method	No. of cases	Platelet adhesiveness
Haemophilia	Wright (1946)	Wright	4	*Decreased*
	Weiner *et al* (1948)	"	2	Normal
	Moolten et al (1949)	Moolten & Vroman	3	*Increased*
Acute coronary thrombosis	Weiner *et al* (1948)	Wright	[illegible]	Normal
	Moolten *et al* (1949)	Moolten & Vroman	[illegible]	Increased
	Eisen *et al* (1951)	"	12	Normal
	Bobek & Čepelík (1958)	"	6	*Normal*
Dicoumarol-treatment	*Spooner & Meyer* (1944)	*Wright*	33	Decreased
	Wright (1945)	"	12 (rabbits)	*Decreased*
	Weiner *et al* (1948)	"	[illegible]	*Decreased*
	Moolten *et al* (1949)	Moolten & Vroman	[illegible]	Normal
	Eisen *et al.* (1951)	"	[illegible]	Decreased

reports indicate that the two methods do not measure the same property of the platelets, and they also suggest that the methods are unreliable.

Except for the investigations of Wright (1941) who studied the influence of anticoagulants, practically no basic studies have been reported on platelet adhesiveness. Thus, there was a lack of knowledge about the behaviour of platelets exposed to foreign surfaces. Such knowledge is necessary for a proper estimation of the adhesiveness of platelets *in vitro*. In the present work, the conditions influencing platelet adhesiveness were studied. Results, based on these investigations, led to the strict standardization of the method, for example, with regard to the influence of red cells on platelet adhesiveness. Thus, it is believed that the present method is technically superior to the earlier methods

This technical superiority does not necessarily imply that the method is better than the earlier ones in other respects The main question for all *in vitro* methods is· Does the test reflect the events occurring *in vivo*? It has been shown in the earlier methods that platelet adhesiveness is increased in patients after parturition and surgical operations (Wright 1942), and in patients with venous thrombosis (Bobek & Čepelák 1958). However, this is not proof that there is any correlation between the adhesiveness *in vivo* and that which is measured by the methods of Wright and of Moolten & Vroman, since we do not know whether thromboembolic disorders are caused, or followed, by a general increase of the adhesiveness of the *circulating* platelets We have, at present, no method of measuring increased platelet adhesiveness *in vivo*, and therefore cannot correlate observations *in vivo* with *in vitro* studies

A correlation between *hypoadhesiveness* of platelets *in vivo* and *in vitro* may be attempted for the following reason. In some haemorrhagic disorders there is a prolonged bleeding time in spite of a normal platelet count If vascular abnormalities can be excluded, the prolonged bleeding time is probably caused by impaired formation of the platelet plugs, which is believed to reflect a defective platelet adhesiveness *in vivo*. The demonstration of a reduced adhesiveness of platelets from these patients *in vitro* would support the idea that there is a correlation between the adhesiveness *in vivo*, and that which is measured by the present method *in vitro*

Moolten *et al.* (1949 c), studying a few patients with haemorrhagic disorders, found a rough inverse correlation between the bleeding time and the number of adhesive platelets They found, however, one instance of a normal bleeding time in a patient with 8,000 platelets per c mm, and also one patient who had a prolonged bleeding time with 120,000 adhesive platelets per c.mm.

In the selected patients studied in this work (pp. 93-98) a fairly good inverse correlation between the prolonged bleeding time and the number of adhesive platelets was found. Therefore the method used probably gives a correct picture of the adhesiveness of platelets *in vivo*, at least with respect to hypoadhesiveness.

2. THE RELATION BETWEEN ADHESIVENESS AND *AGGREGATION* OF PLATELETS

Lundevall (1958, p. 24) found that unspecific platelet clumping (aggregation) was pronounced at room temperature but almost abolished at 37° C at pH 7.2-7.4. In the present work it is shown (p. 28) that the "immediate" adhesion of platelets is not influenced by *temperature* variations between 0° C and 37° C. Therefore, the mechanism of unspecific *platelet clumping probably is not identical to the mechanism* which provokes "immediate" adhesion of platelets to foreign surfaces.

Braunsteiner (1955) also stated that the mechanisms leading to adhesion and to aggregation of platelets were different. Investigations in the present work, however, indicate that the two mechanisms may be related. *Thus, it was found (pp. 82-83) that factor* R, *which in lower concentra*tions provoked only platelet adhesion, in greater concentrations also induced aggregation of the platelets

3. *THE MECHANISM OF THE "IMMEDIATE" ADHESION OF* PLATELETS TO FOREIGN SURFACES *IN VITRO*

Several theories have been proposed to explain the mechanism of platelet adhesion to foreign surfaces and to an injured vessel intima Sawyer *et al.* (1953 a, b, c) believe that platelet thrombus formation is a result of alterations in the electrical charge of the injured intima If this was the whole explanation, however, *it would be difficult to explain* why the initial thrombus consists solely of platelets, since red cells and granulocytes are negatively charged like the platelets, or even more so (*Abramson 1928*). Further, it has been found that platelets stored *in vitro* for more than a few hours do not have haemostatic effect in thrombocytopenic patients (Discussion on *transfusion* of platelets and platelet substitutes Blood 1958, *13*: 1092-1093), in spite of their unchanged negative electrical charge (Abramson 1928). *In vitro*, it has been *demonstrated* that platelets adhere to

quartz particles (Bounameaux 1957), although both the platelets and the quartz particles are negatively charged (Abramson 1928). The theory of Sawyer *et al.* therefore cannot explain the mechanism of platelet adhesion *in vivo* and *in vitro*.

Loeb (1906) found that platelets in lower animal species became morphologically changed on contact with a foreign surface, and adhered to the foreign surface. This theory does not hold for human platelets, because human platelets do not adhere to glass beads in the absence of red cells (pp. 61-62). It should be pointed out that "platelets" in lower species are morphologically (and probably functionally) quite different from human platelets.

Roskam (1922, 1923) believed that the adhesion of platelets to a foreign surface occurred when the surface was "opsonized", *i. e.* changed in some way by contact with plasma. He found, however, that the platelets were still adhesive after heating or after treatment with sodium fluoride or cocaine. His theory therefore does not explain why only uninjured, freshly collected, *i. e.* viable, platelets have haemostatic effect *in vivo* (*vide supra*).

Wright (1945) thought that platelets became adhesive as the result of the deposition of a fibrin film on their surface This theory is contradicted by Pinninger & Prunty (1946), who found a normal bleeding time and normal platelet adhesiveness in a patient with complete afibrinogenaemia.

Bounameaux (1958) found that thrombin formation together with certain co-factors was responsible for platelet adhesion *in vitro*. This theory, however, does not explain why platelets *immediately* adhere to foreign surfaces, because activation of the coagulation factors needs at least several seconds.

From the present investigations, a new theory of the mechanism of platelet adhesion *in vitro* was developed as follows: Platelets are non-adhesive in citrated plasma, but they are adhesive in citrated blood. A substance which makes platelets adhesive can be extracted from red cell ghosts; it is dialysable, probably of anion nature, heat stable, and it needs a co-factor of cation nature (probably calcium) This factor is provisionally called factor R. Factor R has no effect on platelets stored for 24 hours at room temperature, which indicates that in order to be adhesive, the platelets must also be viable Factor R is liberated by contact of red cells with a foreign surface.

The influence of red cells on platelet adhesiveness *in vitro* in fact, was observed by Brecher & Cronkite (1950) In thin preparations of diluted

blood, they found that haemolysis occurred, and the platelets usually flattened out and adhered to the glass. In contrast, freely floating forms were found in thick areas of the preparation where no haemolysis occurred. The authors did not, however, pursue this observation.

4. THE MECHANISM OF PLATELETS ADHESION *IN VIVO*

If it could be shown that factor R was present in the vascular wall and was liberated by injury, the formation of a platelet plug *in vivo* would be explained logically. An attempt to demonstrate factor R in the aortic wall of a cow was, however, unsuccessful. This negative finding could be explained in three ways: 1) The substance is present in the vessel wall, but it is inactivated or lost during the preparative procedures. This is improbable for reasons discussed on p. 71. 2) Factor R is *not* present in the aortic wall, but may be so in other vessels, particularly in the smaller ones. Investigations by Houlihan (1947) on the adhesiveness of platelets to bacteria indicated that an adhesion-promoting substance was present in the walls of veins, but not in arteries. 3) Factor R is not present in the vessels Further studies on this point have not yet been done.

Some observations in the present work as well as those of earlier authors indicate that red cells influence haemostasis, and therefore in all probability influence the adhesiveness of platelets *in vivo*. Based on observations of the effect of direct blood transfusions on the bleeding time in three anaemic and thrombocytopenic children, Duke (1910) claimed that not only platelets, but also red cells influenced the haemostasis He found that the transfusions were followed by a transient rise in the platelet count, which returned to the pretransfusion level after three days. The bleeding time decreased as the platelet number was elevated, and increased again as the platelet count fell. When the platelet count reached the pretransfusion level, however, the bleeding time was still about 50 per cent shorter than before the transfusion. The nature of the influence of red cells on the bleeding tendency (and therefore probably on platelet adhesiveness *in vivo*) is not yet clarified, but some observations indicate that factor R is liberated from red cells at the point of vessel injury. Hugues (1953) found that a zone of haemolysis was always found at the base of the platelet plug *in vivo*. In the present work, it is shown (p. 66) that the haemolysate from red cells contains large amounts of factor R Therefore, it is possible that a local liberation of factor R may occur when a vessel is injured

5. THE EFFECT OF FACTOR R *IN VIVO*

The injection of a preparation containing factor R into rabbits caused an abrupt decrease in the number of circulating platelets, but the platelet count rose to about 70 per cent of normal within 20-30 minutes. It is therefore improbable that the effect of the injection was a rapid destruction and a compensatory formation of new platelets. Most likely, factor R induced an increased platelet adhesiveness, with a temporary sequestration of platelets in the lungs and other organs. When the effect of factor R disappeared, the trapped platelets probably were released into the circulation. The *in vivo* inactivation of factor R may be compared to the inactivation which occurs when factor R is incubated with plasma *in vitro*.

There are several pathologic conditions which have intravascular haemolysis as one of their manifestations. These conditions are often accompanied by a haemorrhagic diathesis and/or thrombosis. After massive transfusions of compatible blood, thrombocytopenia and abnormal bleeding are often observed. The mechanism of the thrombocytopenia in the patients of Krevans *et al.* (1955) could not be explained as the result of dilution of the recipients' blood, by transfusion of non-viable platelets, or by the influence of isoagglutinins in the blood of the donors. In the light of the present investigation, the thrombocytopenia in these patients could be due to the effect of factor R, which is released during the extracorporeal handling of the blood. This mechanism could also be responsible for the thrombocytopenia which develops during procedures which use extracorporeal circulation of blood.

When incompatible blood is transfused, the thrombocytopenia is generally quite severe, and is often accompanied by a marked bleeding tendency. Hardaway *et al* (1956) and McKay *et al* (1956) found that the transfusion of incompatible (human) blood into dogs caused thrombocytopenia, a haemorrhagic diathesis, and widespread thrombosis of small vessels. The thrombi consisted of platelets or a mixture of platelets, fibrin, and granulocytes. If the dogs survived, the thrombi disappeared more rapidly than spontaneously occurring thrombi. The authors suggested that the thrombus formation was caused by a thromboplastinogen-like substance (Quick *et al.* 1954), but the reversibility of the thrombotic condition makes it more likely that the effect was caused by the liberation, and subsequent inactivation, of factor R during intravascular haemolysis.

A similar mechanism may possibly explain the thrombocytopenia accompanying paroxysmal nocturnal haemoglobinuria (Wintrobe 1951, p. 594) and malaria (Diggs 1944).

In congenital haemolytic anaemias, there is no haemoglobinaemia (Crosby & Dameshek 1951), and factor R is therefore probably not released intravascularly. This may explain the general absence of thrombocytopenia (Wintrobe 1951, p. 603) in this condition.

6. THE INACTIVATION OF FACTOR R IN PLASMA

As already mentioned, factor R is rapidly inactivated in platelet-poor plasma *in vitro*. If this inactivation *in vitro* had not been demonstrated, the effect of factor R *in vivo* could not have been postulated. The presence of this inactivating mechanism in addition to the fibrinolytic system may explain why a thrombus does not grow indefinitely.

7. CONCLUDING REMARKS

In the present work I have demonstrated the influence of red cells (factor R) on the platelet adhesiveness *in vitro*. This has led to a new theory for the mechanism of the immediate adherence of platelets to foreign surfaces *in vitro*. Further, some observations have been presented, which indicate that factor R may play a role both in haemostasis and in thrombus formation, but the proof of this theory is still lacking. The problem is, however, of great importance, and further work along these lines should be done. An attempt to solve the problem requires an *in vivo* study of haemostasis and thrombus formation. This could be done by investigating the platelet plug as it forms in mesenteric vessels of rabbits, and such studies are in progress.

Summary

Chapter I gives a brief introduction of the mechanism of haemostasis and thrombus formation The role of platelets and of coagulation is discussed. The theories for the formation of the platelet thrombus are mentioned, and the importance of platelet adhesiveness in the early stage of these processes is stressed. Little was known of platelet adhesiveness, primarily because the methods used for the study of this phenomenon have not been adequate The present work is an *in vitro* study of platelet adhesiveness. Its principal aim has been to develop a satisfactory method for the measurement of platelet adhesiveness *(in vitro)*, and to characterize some of the factors which influence this adhesiveness.

Chapter II describes the materials and methods which have been used, and gives important definitions.

Chapter III is devoted to the measurement of platelet adhesiveness The earlier methods are reviewed, and their methodological errors are emphasized The principle of the present method is simple The number of adhesive platelets is equal to the number of platelets which are lost when the sample is allowed to pass through a glass filter under carefully standardized conditions Reasons are given for the use of a modified Nygaard's method for platelet counting, and the counting error of this method has been evaluated The practical procedure for the measurement of platelet adhesiveness is described in detail, and the accuracy of the method is discussed and documented

Variations in the experimental conditions of the method have been studied systematically The platelet adhesiveness of *citrated blood* was stable for 15-25 minutes after withdrawal of the blood. The number of platelets which adhered to the glass filter increased slightly when the contact time was prolonged, and markedly when the area of the glass filter was increased. The standardization of this area is therefore an important technical point. The platelet adhesiveness decreased with increasing concentrations of citrate, oxalate, and EDTA, whereas increasing concen-

trations of heparin had little influence. Addition of Triton WR-1339 to the blood sample, and silicone coating of the foreign surface reduced the number of platelets adhering to the surface of the filter. Temperature variations between 0° and 37° C did not influence the adhesiveness significantly. The percentage of adhesive platelets was not influenced by the number of platelets in the sample, but, surprisingly, increased proportionally with the number of *red cells* in the sample. The platelet adhesiveness was not influenced by meals.

The platelet adhesiveness in freshly prepared *citrated platelet-rich plasma* was nearly zero. However, during storage for a few hours at 4° C the adhesiveness increased from 0 to about 60 per cent. Simultaneously, the total platelet count decreased in the samples. This "cold-induced" adhesiveness disappeared if the platelet-rich plasma was further incubated for 24 hours at 20° C. Storage at 20° C for 48 hours followed by storage at 4° C did not increase the adhesiveness, suggesting that the platelets have been changed irreversibly during storage at 20° C for 48 hours. The "cold-induced" adhesiveness was due to changes in the platelets and not to changes in the plasma. It was inhibited by increasing concentrations of anticoagulants. This "cold-induced" adhesiveness of platelets should be considered in all platelet work, including platelet transfusions. Procedures which involve manipulations of platelets in the cold for more than 1-2 hours should be avoided, since they lead to great losses of platelets. Storage of platelets in plasma at 20° C for more than about 5-6 hours should also be avoided.

Platelets resuspended in *saline* are adhesive EDTA does not abolish this adhesiveness, as opposed to its effect on the adhesiveness of platelets in whole blood and in platelet-rich plasma which has been stored in the cold. The adhesiveness of the platelets in saline, therefore, is different from the adhesiveness of platelets in their natural environment, and is possibly the result of an artifact.

Chapter IV gives the normal range for platelet adhesiveness in citrated blood and plasma. In blood, the number of adhesive platelets varied between 52,000 and 157,000 with a mean of 90,000 per c mm The percentage of adhesive platelets varied between 26 and 68, with a mean of 42 per cent. In plasma, the number of adhesive platelets varied between -17,000 and +65,000, with a mean of 15,000 per c mm. The percentage of adhesive platelets varied between -4 and +15, with a mean of 4 per cent.

Chapter V deals with the influence of red cells on platelet adhesiveness. It is shown that a substance (factor R) is released from the red cells by contact with a foreign surface. In the presence of this substance the non-

adhesive platelets in citrated platelet-rich plasma become adhesive. Factor R could not be washed from the red cells with saline, nor could it be removed by trypsin. If red cells were lysed and the ghosts removed by centrifugation, the supernatant contained factor R activity. However, the ghosts were also very active, suggesting that factor R is located on the red cell stroma, but is partially released to the surrounding medium by haemolysis.

Factor R was heat-stable, dialysable, and was stable for months when stored at -25° C. It was adsorbed by an anion exchange resin, but not by a cation exchange resin. Its effect could be counteracted by high concentration of calcium-binding anticoagulants. When increasing amounts of factor R were added to citrated platelet-rich plasma, the adhesiveness increased, within certain limits, in proportion to the logarithm of the concentration of factor R. In high concentrations factor R provoked macroscopic aggregation of platelets. It acted only on "viable" platelets Thus, it did not increase the adhesiveness of platelets stored in plasma at 20° C for 24 hours. Factor R was inactivated by storage at 20° C in platelet-poor plasma

Preliminary experiments on the *in vivo* effect of factor R showed that it caused a pronounced but transient decrease of the circulating platelets when it was injected intravenously into rabbits.

Factor R activity was not found in saline extract of human brain (tissue thromboplastin), in cephalin, or in saline extracts from bovine aorta. Other tissues were not examined

Chapter VI deals with the platelet adhesiveness in various bleeding disorders Platelets in citrated blood from patients with thrombasthenia were non-adhesive, and factor R or storage at 4° C had no effect on these platelets. In saline suspensions, however, the platelets became normally adhesive The platelet adhesiveness was normal in 14 of 15 patients with von Willebrand's disease. The percentage of adhesive platelets was also normal in eight of nine patients with thrombocytopenia. In congenital and acquired coagulation disorders the platelet adhesiveness was normal, suggesting that the clotting factors play a minor role in the so-called "primary haemostasis" A patient with pernicious anaemia, who had a prolonged bleeding time and a reduced total platelet count, also had a relative decrease of adhesive platelets The bleeding time became normal four weeks after treatment with vitamin B_{12} had started. The findings suggest that the prolongation of the bleeding time was due to the decreased adhesiveness of the platelets, rather than to the reduction in the total platelet count Similar observations were made in other patients

Chapter VII discusses the main points in this investigation. The first part of the discussion deals with methodological problems. The discrepancies between the results obtained with the present method and those obtained with other methods suggest that there are qualitative differences between the properties which are measured by the different methods. Based on the observations made with the present method a new theory is suggested for the mechanism of the immediate adherence of platelet to foreign surfaces *in vitro*. Platelets in citrated blood are assumed to be non-adhesive until a substance (factor R) is released from the red cells by contact with a foreign surface. In the presence of this substance "viable" platelets become adhesive. Observations in bleeding disorders suggest that the *in vitro* adhesiveness is releated to the haemostatic properties of the platelets *in vivo*.

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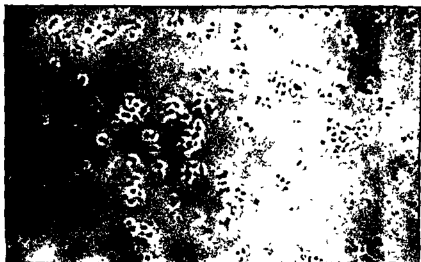
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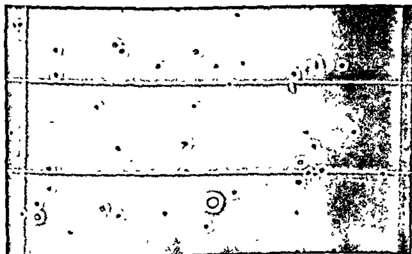
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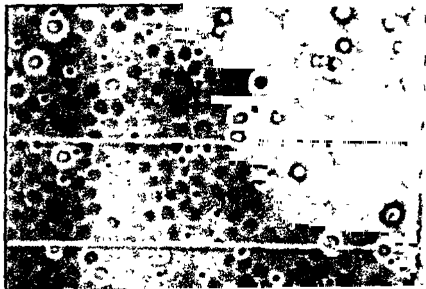
Microphotographs



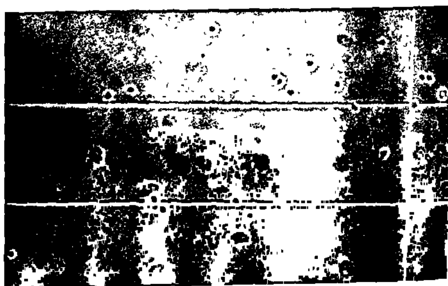
No 1 *Platelets adhered to a plane glass surface*
Two drops of citrated whole blood were tilted on a cover slip for 4 minutes The blood was then washed off with platelet-poor citrated plasma (160 ×)



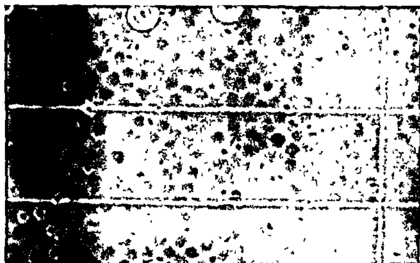
No. 2 *Platelets in a counting chamber Nygaard's method*
(Dilution 1 20) (160 ×)



No. 3 *Platelets in a counting chamber: method of Feissly & Ludin.*
(Dilution 1 : 20) (160 ×)



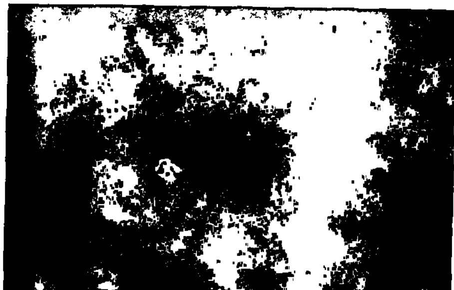
No. 4 *Platelets in a counting chamber: method of Brecher & Cronkite*
(Dilution 1 : 100) (160 ×)



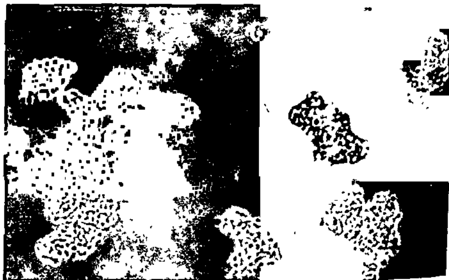
No. 5 *Platelets in a counting chamber, method of Brecher & Cronkite*
(Dilution 1 20) (160 ×)



No 6 *Platelet clumping caused by addition of one drop of factor R (diluted 1 3) to 1 ml of citrated platelet-rich plasma* (64 ×)



No 7 *Absence of platelet adherence to glass surface when platelet-rich citrated plasma has been in contact with plane glass surface.* Citrated platelet-rich plasma was tilted on a cover slip for 4 minutes, then washed off with platelet-poor citrated plasma. (160 ×)



No 8 *Clumped platelets adhered to glass in the presence of factor R* Citrated platelet-rich plasma, to which factor R had been added to a final dilution of 1 250, was tilted on a cover slip for 4 minutes, then washed off with citrated platelet-poor plasma *containing the same amount of factor R (160 ×)*